EVALUATION OF BOVIE GAMMA-INTERFERONE AND TUBERCULIN TEST FOR THE DIAGNOSIS OF BOVINE TUBERCULOSIS

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ABSTRACT

The study was conducted in 3 farms in Behera, Matrouh and Dakahlya provinces. These organized farms did not have previous records on animals with confirmed *M. bovis* infection as the herd had been previously skin tested. A total of 1323 animals of 1 year old and above were included in the study. All animals under our experiment were examined using Tuberculin Test; the positive cases were examined using T.B. Feron Test. The positive cases in both tests were slaughtered for PM and some samples were taken for bacteriological examination on specific media. Confirmation of the isolates by real time PCR. The results revealed that the number of total tested cattle by tuberculin were 1323 the positive were 357 (27 %) by tuberculin test, then tested by bovine gamma-interferon (γ-IFN) enzyme immunoassay, the positive were 212 (16 %), in the first farm the tested cattle were 120 animals and the positive were 79 (6 %) by tuberculin test, then tested by bovine gamma-interferon (γ-IFN) enzyme immunoassay, the positive were 46 (3.5 %), the second farm the tested cattle were 520 and the positive reactors for tuberculin test were 274 (20.7 %), then tested by bovine gamma-interferon (γ-IFN) enzyme immunoassay, the positive were 166 (12.5 %) and the third farm the tested cattle were 683 and the positive cases were 4 animals (0.3 %) by tuberculin test, then tested by bovine gamma-interferon (γ-IFN) enzyme immunoassay and all the samples gave negative results. It is important to increasingly focus resources to target control strategies based on more effective diagnostic methods so the usage of T.B. Feron for detection of T.B. is more useful. And also the usage of T.B. Feron test to differentiate between vaccinated and infected cattle.

Key words: Tuberculosis, Gamma Interferone, Cattle, Elisa

INTRODUCTION

Tuberculosis (TB) is a major health problem throughout the world and represents the most frequent cause of death for a single infectious agent worldwide (Khalili *et al.,* 2010). It is a chronic debilitating disease in cattle, but it can occasionally be acute and rapidly progressive. In the late stages, common symptoms including progressive emaciation, low–grade fluctuating fever, weakness, and inappetence are observed. Animals with pulmonary involvement usually have a moist cough that is worse in the morning, during cold weather or exercise, and may have dyspnea or tachypnea (Une and Mori, 2007). Infection leads to a decrease in milk production (10-20%), loss of weight, reduction of fertility and condemnation of carcasses of infected animals (Collins, 2006).

Latent tuberculosis infection (LTBI), a non-communicable asymptomatic condition, might develop tuberculosis disease months or years later. The main purpose of LTBI diagnosing is to consider medical treatment for preventing tuberculosis disease (Ferrara *et al.,* 2005).

Several methods have been used for the diagnosis of bovine TB (BTB), such as direct detection of the etiologic agent in biological material (for instance; culture and molecular assays), or in the indirect detection through the identification of a host immune response to the etiologic agent (for instance; tuberculin test and interferon-gama) (De la Rua-Domenech *et al.,* 2006; Schiller *et al.,* 2010).

Isolation of *M. bovis* is considered the "gold standard" for BTB diagnosis. However, the long period required for the isolation and biochemical identification, is a limitation because it may require more than twelve weeks to complete the final diagnosis, and also low sensitivity (Collins *et al.,* 1994). The collected samples are subjected to decontamination by the addition of NaOH, H_2SO_4, oxalic acid, or quaternary ammonium compounds, to eliminate competitive microorganisms (Ambrosio *et al.,* 2008; Medeiros *et al.,* 2010;Young *et al.,* 2005).

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Unfortunately, the toxic effects of these substances may affect mycobacterial viability, thereby interfering with culturing the organism.

The immunological diagnosis of BTB is based on delayed-type hypersensitivity (DTH) reaction *in vivo*, represented by the tuberculin skin test (TST) (Schiller *et al.,* 2010). This is an indirect method of diagnosis of TB and can reveal incipient infections, with three to eight weeks after contact with the *M. bovis*. It is a widely used test recommended by Robert Koch in 1890 (Monaghan *et al.,* 1994). However, it has low sensitivity and in individuals vaccinated with Bacillus Calmette-Guerin (BCG), it is often associated with a false-positive result due to cross-reactive immune responses to antigens common to MTB. The response of T-cells to these proteins is a measurable secretion of interferon γ (IFN-γ) (Gursimran *et al.,* 2016).

The T.B. FERON test is a test for Cell Mediated Immune (CMI) responses to peptide antigens that simulate mycobacterial proteins. These proteins (ESAT-6, CFP-10, and TB7.7) are absent from all BCG strains and from most non-tuberculous mycobacteria with the exception of *M. kansasii, M. szulgai* and *M. marinum*. Individuals infected with *M. tuberculosis* complex organisms (*M. tuberculosis, M. bovis, M. africanum, M. microti, M. canetti*) usually have lymphocytes in their blood that recognize these and other mycobacterial antigens. This recognition process involves the generation and secretion of the cytokine, IFN-γ. The detection and subsequent quantification of IFN-γ forms the basis of this test (Ferrara, 2005).

Molecular biology techniques are widely applicable to laboratory diagnosis of infectious diseases caused by bacteria (Cortez *et al.*, 2006). Faster diagnosis of TB are of significant clinical importance, therefore, PCR have been proposed for the rapid detection of small amount of *M. bovis* DNA (de Kantor and Ritacco, 2006). This study aimed to evaluate the use of both Tuberculin test and T.B. Feron for the diagnosis of bovine tuberculosis in different localities. The utilization of T.B. FERON test to differentiate between vaccinated and infected cattle was assessed.

MATERIALS AND METHODS

Animals
The study was conducted on 3 farms in Behera, Matrouh and Dakahlya Governorates. The farms included in the study had no previous records on animals with confirmed *M. bovis* infection because the herd was previously tested with skin test. A total of 1323 animals of one year old and above were included in the study. Verbal approval from the farm owners was taken for the participation in the study.

Tuberculin test
Mammalian PPD tuberculin was prepared by Bacterial Diagnostic Products Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbassia, Cairo, Egypt. All the animals were subjected to comparative cervical intradermal tuberculin test as per the guidelines from the World Organization for Animal Health (OIE). Briefly, the test was carried out in the middle third of the neck of each animal where avian tuberculin PPD-2500 (PPD-A) (Prionics) and bovine tuberculin PPD-3000 antigens (PPD-B) (Prionics) were injected (i.e., 0.1 ml of PPD) in two sites of neck 12 cm apart. Skin thicknesses were measured with caliper before and 72 hour after PPD injections. The result is expressed as:

(A) Positive reactor: Difference of the skin thickness at the injection sites is at least 4 mm or greater, (B) negative reactor: No reaction to the bovine antigen or the difference of the skin thickness at the injection sites does not exceed 2 mm, while (C) inconclusive reaction: Reaction to both PPD-B and PPD-A exceeded 2 mm, but the difference between the bovine and avian reaction was <4 mm. (Awah-Ndukum, 2016).

T.B FERON Test
Blood samples from tuberculin positive animals (n=357) were collected from the jugular vein in commercially available sterile 10 ml heparinized tubes, heparin as anti-coagulant and gentle mix blood by inversion several times to dissolve the heparin.

Blood samples should be transported to the laboratory at room temperature (22±3℃, avoid extremes). The immunoassay was performed using T.B. FERON- kit from (BIONOTE, Inc., USA) according to the manufacturer's instructions. Briefly, in this test system, 1.5 ml aliquots of heparinized blood were dispensed into individual wells of 24-well tissue culture plates and incubated with 100 µl each of stimulating antigens (PPD-B and PPD-A) and PBS (non-stimulating control) for 16-24 h at 37°C in a humidified atmosphere with 5% CO_2. The plasma was then collected and assayed for γ-IFN production in duplicate at OD=450 nm. (Schiller *et al.,* 2010).

Bacteriological examination
Positive animals by both tuberculin and T.B FERON tests were slaughtered for PM and some samples were taken for bacteriological examination on specific media. Lymph node samples were initially cut into cubes (5 mm×5 mm). Cubed lymph node sample (approx.10 g) was ground with a small amount of sterile sand using a mortar and pestle before the addition of 12 ml 5% oxalic acid (Stewart *et al.,* 2013). The sample was mixed again and the liquid content transferred to a centrifuge tube, discarding the remaining solid material from the mortar. The sample was transferred to a rotary mixer and mixed for 15 min at 37°C, followed by centrifugation at 1.620×g

for 15 min. The supernatant was discarded and the sediment washed twice in sterile phosphate buffered saline (PBS). Using a sterile swab, the sediment was streaked directly onto Löwenstein-Jensen media. The samples were incubated at 37°C for 90 days with weekly examination. The isolation of *M. bovis* was carried out in a class II biological safety Type A2 cabinet.

Molecular identification by real time PCR
Suspected colonies were identified by performing a real time PCR using MTplexdtec-RT-qPCR Test (Akhtar

et al., 2015). That comprises a series of species-specific targeted reagents designed for detection of all species contained in the *Mycobacterium tuberculosis* complex. DNA extraction from the isolates was performed using Bacterial DNA extraction kit (BioTeke Corporation, Shanghai, China) according to the manufacturers' guidelines. Extracted DNA from the suspected cultures was subjected to real-time PCR using MTplexdtec-RT-qPCR Test. The primers and TaqMan probe target a sequence conserved for all strains of each single species belonging to *Mycobacterium tuberculosis* complex.

RESULTS

Table 1:

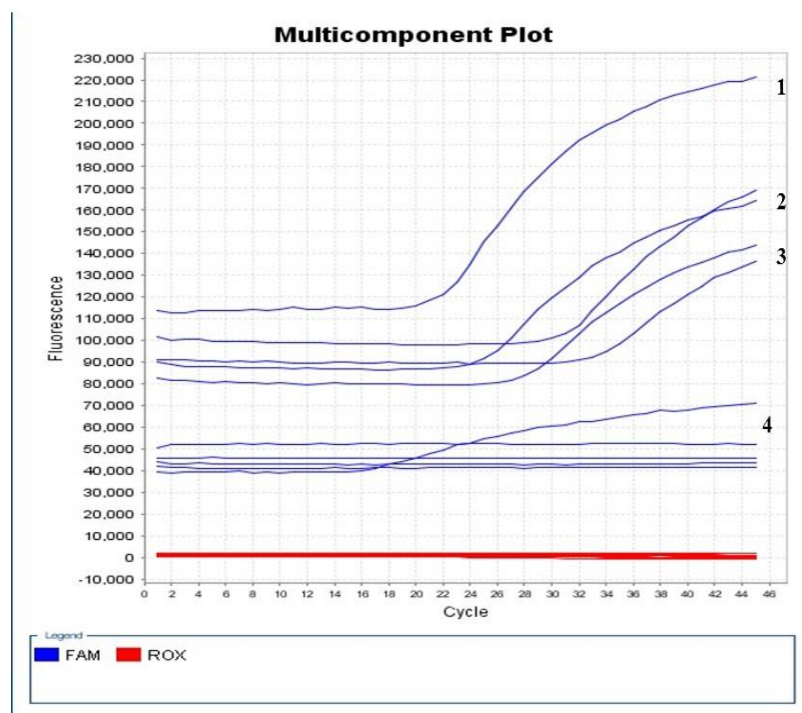| Farm No	Total No. of Animals tested tuberculin	Positive Tuberculine test		positive T.B. FERON test	
		No.	*%*	*No.*	*%*
Farm 1	120	79	6	46	3.5
Farm 2	520	274	20.7	166	12.5
Farm 3	683	4	0.3	0	0
Total No.	1323	357	27	212	16

Figure 1: Amplification curve of *Mycobacterium* complex rtPCR (1: undiluted positive control, 2: duplicate of 2 fold dilution of sample1, 3: duplicate of 2 fold dilution of sample 2, 4: five reactions of negative controls).

DISCUSSION

The results in Table (1) revealed that out of 1323 cattle tested, 357 (27%) were positive by tuberculin test. The results of bovine gamma-interferon (γ-IFN) enzyme immunoassay, revealed 212 (16 %) positive animals. in the first farm the tested cattle were 120 animals and the positive were 79 (6 %) by tuberculin test, then tested by bovine gamma-interferon (γ-IFN) enzyme immunoassay, the positive were 46 (3.5 %), the second farm the tested cattle were 520 and the positive reactors for tuberculin test were 274 (20.7 %), then tested by bovine gamma-interferon (γ-IFN) enzyme immunoassay, the positive were 166 (12.5 %) and the third farm the tested cattle were 683 and the positive cases were 4 animals (0.3 %) by tuberculin test, then tested by bovine gamma-interferon (γ-IFN) enzyme immunoassay and all the samples gave negative results.

Since 2006, the γ- IFN assay is an assay through which it is possible to verify the existence of cell-mediated immune response developed by the body of the animal in response to mycobacterial infection. γ-IFN produced by T- lymphocytes of the infected animal is detected, using monoclonal anti-γ IFN. The lack of detection of γ-IFN characterizes the negativity of the animals to infection *M. bovis* since lymphocytes from uninfected cattle do not produce this cytokine in specific ways. As this is an *in vitro* test that has the advantage of not interfering with the immune status of the animal and may be repeated in the same animal is the need to respect the period of desensitization. This assay showed the increase in the sensitivity and the possibility of more rapid repeat testing, no need for a second visit to the farm and more objective test procedures and interpretation in comparison to the tuberculin test (Faye *et al.,* 2011; Neill *et al.,* 1994; Schiller *et al.,* 2010).

The strategic application of the γ IFN assay, as an adjunct to the tuberculin test, can facilitate the early removal of infected animals in herds. The objective of the assay is to identify high-risk animals that are potentially infectious for other cattle can generate confidence in herd-owners that rational decisions can be made based on sound scientific principles, and that effective schemes can be devised to make more rapid progress in the elimination of the infection from affected herds (Schiller *et al.,* 2010).

The assay is based on the release of γ IFN from sensitized lymphocytes during a 16-24 hours incubation period with specific antigen and makes use of comparison of γ IFN production following stimulation with avium and bovine PPD (Alito *et al.,* 2003; de la Rua-Domenech *et al.,* 2006; Schiller *et al.,* 2010; Vordermeier *et al.,* 2008). *M. tuberculosis* complex specific antigens have also been used to improve γ IFN assay specificity, especially in population groups testing positive to the tuberculin test. The use of these antigens may also offer the ability to differentiate BCG-vaccinated from unvaccinated animals. (Faye *et al.,* 2011; Fentahun and Luke, 2012).

Our results coincide with that reported by Ryan *et al.* (2000) who reported that, in cattle, the sensitivity and specificity of the gamma- IFN test for diagnosis of *M. bovis* was 85 and 93%, respectively, when compared with the caudal fold test. No significant differences in the sensitivity and specificity of the test were observed between blood samples that were cultured on the day of collection and those cultured the day after collection. These findings support the use of the gamma -IFN test as a practical test that can be used to complement the caudal fold skin test.

The results of bacteriological examination of lymph nodes from positive cases with both T.B. FERON and tuberculin tests revealed that all the samples were positive by culture technique and the identified species was *M. bovis*. Confirmation of the isolates by real time PCR showed positive reactions (Figure 1).

In accordance, Tyler *et al.* (2011) reported that culture of *M. bovis* from diagnostic specimens is the gold standard for the diagnosis of bovine tuberculosis in USA. Detection of *M. bovis* by PCR in tissue homogenates may provide a simple rapid method to complement bacterial culture. A significant impediment to PCR based assays on tissue homogenates is specificity since mycobacteria other than *M. bovis* may be associated with the tissues. Also, Wessam Youssef *et al.* (2017) examined a total of 49 specimens collected from four major abattoirs (El-Basateen- El-Monieb- Beni-Suef-Al-fayoum) to be analyzed bacteriologically and biochemically for the isolation, identification and confirmation of *M. bovis* with molecular methods. Only 19 isolates were found to be positive slow-growers Mycobacterium species by conventional cultivation method on solid medium and were identified biochemically to 17 *M. bovis* isolates and 2 isolates *M. tuberculosis*.

Despite all the efforts to control BTB, the disease persists, with serious implications. This zoonotic disease constitutes a significant economic burden to the agricultural industries and for human health. Eradication programs based on tuberculin testing and subsequent slaughter of positive animals have been reported successful in many developed countries. However, tuberculin test is limited in its specificity and sensitivity, therefore, culture should be used to confirm the presence of *M. bovis*. Molecular techniques such as PCR can also detect *M. bovis* directly in clinical samples. Many factors contribute to the persistence of BTB, such as the limitations of diagnostic tests (concerning both sensitivity and specificity), larger herd sizes, increase

in animal movements and trade, and limited options for control, such as limitations on whole herd depopulation (Wessam Youssef *et al.,* 2017).

CONCLUSION

In conclusion, considering the current trends associated with BTB control and eradication programs, it is important to increasingly focus resources to target control strategies based on more effective diagnostic methods. Hence, the use of TB-Feron for detection of BTB is more useful.

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تقييم اختبار الجاما ـ انترفيرون والتيوبيركلين فى تشخيص مرض السل البقرى

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تمت هذه الدراسة فى ثلاث مزارع فى محافظات البحيرة ومطروح والدقهلية. وهذه المزارع مؤسسة بأحدث التقنيات فى ادارتها ولم يكن لديها تقارير سابقة بأصابة الابقار بمرض السل البقرى وأيضاً لم يتم فحص القطيع بأختبار التيوبركلين. تم أجراء الدراسة على عدد ١٣٢٣ حيوان تتراوح أعمارهم من عام فأكثر. تم فحص كل الحيوانات بأختبار التيوبركلين. وتم فحص الحالات الايجابية لهذا الاختبار بواسطة اختبار أل. تى. بى. فيرون. وتم ذبح الحالات الايجابية فى الاختبارين وتم الفحص التشريحى وأخذ بعض العينات لأجراء الفحص البكتيريولوجى بأستخدام المنابت الخاصة بميكروب السل، ثم تم اجراء أختبار تفاعل البلمرة الكمى على جميع المعزولات لتأكيد التصنيف البكتيريولوجى. أظهرت النتائج أن اجمالى الحيوانات التى تم فحصها ١٣٢٣ وكانت الحالات الايجابية ٣٥٧ بنسبة (٢٧ %) بأختبار التيوبركلين وتم أختبارهم بأختبار تى. بى. فيرون وكانت الحالات الايجابية ٢١٢ بنسة (١٦ %). ففى المزرعة الاولى كان عدد الابقار التى تم فحصها ١٢٠ بقرة وكانت الحالات الايجابية ٧٩ بنسبة (٦ %) لأختبار التيوبركلين وبفحصهم بأختبار تى. بى. فيرون كانت الحالات الايجابية ٤٦ حالة بنسبة (٣،٥ %). وفى المزرعة الثانية كان عدد الابقار التى تم فحصها ٥٢٠ بقرة وكانت الحالات الايجابية ٢٧٤ بنسبة (٢٠،٧ %) لأختبار التيوبركلين وبفحصهم بأختبار تى. بى. فيرون كانت الحالات الايجابية ١٦٦ حالة بنسبة (١٢،٥ %). اما المزرعة الثالثة كان عدد الابقار التى تم فحصها ٦٨٣ بقرة وكانت الحالات الايجابية ٤ حالات بنسبة (٠،٣ %) لأختبار التيوبركلين وبفحصهم بأختبار تى. بى. فيرون كانت كل الابقار سلبية. وأنه من الآهمية توجيه الموارد فى تحديد استراتيجيات التحكم والوقاية من مرض السل البقرى مبنية على استعمال الآختبار الآكثر دقة وفاعلية للتشخيص ولذلك بتطبيق اختبار تى. بى. فيرون للكشف عن الآصابة بميكروب السل البقرى يكون الآكثر دقة وحساسية للتشخيص. وأيضا بولسطته يمكن التفريق بين الآبقار المصابة والآخرى المحصنة.