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Development, Preparation and Evaluation of a Murine Monoclonal Antibodies-Based Interferon-Gamma Enzyme-Linked Immunosorbent Assay (ELISA) for Rapid and Accurate Diagnosis of Bovine Tuberculosis

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ABSTRACT

Bovine tuberculosis is an infectious and chronic disease affecting cattle, caused by *Mycobacterium bovis* (*M. bovis*). The zoonotic nature of the disease has a serious worldwide impact on human health. Also, the significant economic costs caused by such disease in addition to the deficiency of precise estimates of the actual disease prevalence necessitate more efficient detection and control measures, particularly in developing countries. The main target of the present work was to develop a local, less expensive bovine tuberculosis interferon-gamma ELISA (Bo-IFN-γ ELISA) kit for the rapid and accurate diagnosis of bovine tuberculosis. In the current study, three murine hybridomas producing monoclonal antibodies (mAbs) against bovine interferon-gamma (Bo-IFN-γ) were developed and their monoclonal antibodies were characterized. The produced mAbs were of IgM isotype and their specificity was proved using the western blot technique. The prepared mAbs were used for the development of the bovine IFN- γ ELISA test that was evaluated for laboratory diagnosis of bovine tuberculosis. The sensitivity and specificity of the developed ELISA kit as compared with the standard tuberculin skin test were determined. This method is based on the measurement of IFN-γ released from sensitized bovine lymphocytes upon exposure to the mycobacterial antigens. Using checkerboard titration, the optimal coating concentration of anti- Bo-IFN-γ was 20µg/well. Blood samples from apparently healthy cattle, which proved negative in the tuberculin test, were examined with the developed kit and the cut-off value (COV) was equal to 0.30 optical density (OD). In a preliminary study to evaluate the sensitivity and specificity of the developed ELISA, 23 cattle were examined with the Bo-IFN-γ ELISA and the standard tuberculin skin test. The developed Bo-IFN-γ ELISA showed high sensitivity (98%) and specificity (71.4%) in the diagnosis of bovine tuberculosis as compared to the standard tuberculin skin test.

Key words: Bovine Tuberculosis, Murine Monoclonal Antibodies, Rapid Diagnostics, ELISA, Sero-Diagnosis, Applied Immunology.

INTRODUCTION

The main causative agent of bovine tuberculosis is *Mycobacterium bovis*, which is a member of the *Mycobacterium tuberculosis* clad (MTC), (formerly *Mycobacterium tuberculosis* complex), and based upon 16S ribosomal RNA (rRNA) sequencing studies, it has been established that more than 99.95% identity with other members of MTC (Garnier et al. 2003; Smith et al. 2009). Bovine tuberculosis is a serious disease mainly affecting cattle and a wide range of mammalian hosts and its zoonotic nature has a serious impact on human health (Humblet et al. 2009; Mohamed 2020; Borham et al. 2022; Hussain et al. 2022; Siddique et al. 2022). The disease is still widespread in developing nations, and it has resulted in serious economic losses due to cattle deaths, chronic illnesses, and trade restrictions. Bovine tuberculosis (bTB) may, in certain circumstances, pose a major threat to critically endangered. The bTB is predominantly spread by cattle, hence this animal species is the primary target of eradication initiatives globally (Mohamed 2020; Desouky et al. 2023). Diagnosis of the disease depends mainly on tests that measure the cell-mediated immunity (CMI) developed post-infection and

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by culture-based bacteriological examination and/or molecular-based polymerase chain reaction (PCR) technique (Thomas et al. 2021; Smith et al. 2021; Nasr et al. 2021). The standard method used for the detection of bovine tuberculosis is the tuberculin test, first developed in 1891 by Robert Koch and first introduced in cattle in 1899 by McFadyean (Good et al. 2018). The tuberculin skin test (TST) is an indirect method used for the diagnosis of TB and can reveal incipient infections, within three to eight weeks after contact with the *M. bovis*. The test involves intradermal injection of tuberculin, a poorly defined protein preparation from *Mycobacterium* cultures, into animals and subsequent detection of swelling and induration at the site of infection two or three days later (Good et al. 2018). This test, however, can lack both sensitivity and specificity (Xin et al. 2013; Goodchild et al. 2015; Duignan et al. 2019; Srinivasan et al. 2019; Islam et al. 2020; Carneiro et al. 2021). Additionally, there are a number of known limitations with this test, including challenges with administration and interpretation of data, the requirement for a follow-up visit, a poor level of standardization, and subpar test accuracy. Also, the test is labor intensive and time-consuming. Consequently, many investigators have attempted to develop alternative methods (Good and Duingnan 2011; Broughan et al. 2016; Pucken et al. 2017; Srinivasan et al. 2019; Didkowska et al. 2021; Smith et al. 2021).

The application of serological tests for measuring antibodies against *Mycobacterium* antigens has been tested by previous conducted studies (Chambers et al. 2009; Awah-Ndukum et al. 2012; Ray and Waters et al. 2017; Infantes-Lorenzo et al. 2019; McCallan et al. 2021; Sun et al. 2021). These antibody techniques, however, are of low sensitivity and specificity due to the antigenic cross-reactivity between mycobacteria often encountered by all mammalian species and due to the finding that predominant immune responses to *M. bovis* are cell-mediated in nature.

Other in *vitro* tests that depend upon CMI, e.g., lymphocyte blastogenesis assay has been tested (Dalley et al. 1999). Although this assay showed a comparable sensitivity and specificity to the tuberculin skin test, it is impracticable for testing large numbers of animals and requires a long incubation time (up to 6 days). Several other assays have been described, which depend primarily upon the measurement of interferon-gamma (IFN- γ) released from sensitized lymphocytes upon exposure to the sensitizing antigen(s) (Schiller et al. 2010; Morar et al. 2013; Bezos et al. 2014; de la Cruza et al. 2018; Xin et al. 2018; Chileshe et al. 2019; Steinbach et al. 2019; Martucciello et al. 2020; Xia et al. 2020; Jang et al. 2020). The measurement of IFN-γ can be done using biological assays. Most of these bioassays, however, are time-consuming (4 days), labor intensive, and lack both sensitivity and specificity (Slobbe et al. 2000; Xia et al. 2020; Jang et al. 2020).

The development of monoclonal antibodies against bovine IFN-γ made it easier and simpler to measure IFN-γ produced from stimulated lymphocytes using a sandwich ELISA. Application of ELISA for measuring Bo-IFN-γ showed sensitivity and specificity comparable and even better than the standard tuberculin test (Bass et al. 2013; Neeraja et al. 2014; Genç et al. 2015; Risalde et al. 2017; Keck et al. 2018; Li et al. 2022). IFN-γ assay use was also extended for the diagnosis of human tuberculosis using monoclonal antibodies produced against human IFN-γ (McCallan et al. 2021) and for the diagnosis of Johne's disease in cattle (Fan et al. 2012). ELISA measurement of IFN-γ represents a suitable candidate to diagnose tuberculosis in man and animals and other diseases that involve strong stimulation of CMI. The current study represents a contribution to the efforts of improving the diagnosis and eradication of bovine tuberculosis in developing countries through the production of a relatively cheap and rapid ELISA test that can be applied to a large number of animals. It involves the production of murine hybridomas producing monoclonal antibodies against Bo-IFN-γ and its use in the development of a local less expensive ELISA test for rapid diagnosis of bovine tuberculosis through the detection of bovine interferon-gamma produced by the PPD-sensitized lymphocytes in *vitro.* The sensitivity, specificity, and accuracy of the developed ELISA are determined using the tuberculin test as a gold standard test.

MATERIALS AND METHODS

Ethics Approval

According to the (Animal Research: Reporting of In-Vivo Experiments-ARRIVE) standards, the current study is performed. The institutional animal care and use committee's (IACUC) rules were strictly adhered to throughout the current study's protocols involving the use of animals.

Preparation of Murine Hybridoma Producing Bo-IFN-γ Specific mAbs (Jungersen et al. 2002; Li et al. 2007; Ma et al. 2019; Wood et al. 1990; Bolt and Mahoney 1997; Schiller et al. 2009) as follows:

Antigen Preparation and Mouse Immunization

BALB/C mice were immunized with rBo-IFN-γ (AbD Serotec Company, UK- 25µg/vial). 25µg of the rBo-IFN-γ were homogenously suspended in 1mL PBS (0.01mM pH 7.4) and 200µL of this antigen solution was emulsified in 200µL of complete Freund's adjuvant (SIGMA-ALDRICH) and injected subcutaneously (S/C) using 0.2mL for each mouse. From the prepared rBo-IFN-γ antigen solution 600µL were emulsified in 600µL incomplete Freund's adjuvant (SIGMA-ALDRICH) and 0.2mL of this mixture were injected S/C at 14, 28, and 42 days after the first inoculation. Four days before fusion 20µg of rBo-IFN-γ antigen solution without adjuvant was injected intra-peritoneal (I/P) into the immunized mice prior to removal of the spleen for cell fusion.

Cell fusion, Hybridoma Selection, and Screening

The mice were sacrificed four days after the last vaccine booster, the spleen was taken and minced, and the splenocytes retrieved were combined with P3NS1 myeloma cells at a ratio of 10:1. After 1 minute of warm (37ºC) RPMI medium addition, 50% polyethylene glycol (PEG, MW 1500; SIGMA-ALDRICH) was used to induce cell fusion. Following the PEG treatment, the cells were diluted and plated onto 96-well plates with HAT medium (SIGMA-ALDRICH) supplemented with 20%

FCS. Expanding hybridomas were discovered 7 to 10 days after fusion, and an indirect ELISA was used to check the supernatant for the presence of rBo-IFN-specific mAbs. Then, using limiting dilution, hybridoma cells that secreted rBo-IFN-specific mAbs were propagated, cloned, and kept in liquid nitrogen (-196ºC).

Characterization of the Positive Hybridoma Producing rBo-IFN-γ-Specific Monoclonal Antibodies

Agar gel precipitation testing was used to determine the class of the produced rBo-IFN-specific monoclonal antibodies (mAbs), and mouse immunoglobulin isotyping kits (Mouse Type Isotyping kit, BioRad, Hercules, CA, USA) (Bolt and Mahoney 1997).

Production of ascetic fluid in BALB/C mice

Two BALB/C mice were primed with Pristane (SIGMA-ALDRICH) and injected intraperitoneally with about 106 hybridoma cells seven days later for each hybridoma clone secreting anti-rBo-IFN-mAbs. The mice's ascetic fluid was taken ten days later, centrifuged at a low speed (3,000xg, 10min), titrated using an ELISA, aliquoted, and kept at (-70ºC). We did not employ the hybridoma clones that had low growth to make ascetic fluid.

Preparation of ELISA kit for Measurement of IFN-γ Produced by Bovine Blood Lymphocytes Following Sensitization with PPD (Rothel et al. 1990; Lilenbaum et al. 1999; Wood and Jones 2001; Gormley et al. 2006; Schiller et al. 2009; Ferrara et al. 2009; Faye et al. 2011; Bass et al. 2013; Genç et al. 2015; Risalde et al. 2017; Smith et al. 2021; Li et al. 2022) as follows:

Coating ELISA Microtiter Plates with Bo-IFN-γ mAbs

Murine anti-Bo-IFN monoclonal antibodies (0.5mg/mL) were coated onto Nunc MaxiSorp 96-well flat-bottom polystyrene microtiter plates (Cat. No. 442404) and incubated at 37°C for 1 hour before being incubated at 4°C overnight in the refrigerator. Following two washings with the washing buffer, the plates were blocked with 1% BSA (100µL/well) at 37°C for an hour. After that, three washings with PBS-Tween 20 (0.05%) buffer were performed. After drying, the plates were stored at 4°C until use.

Evaluation of the Sensitivity and Specificity of the Developed ELISA Test as Compared to the Standard Tuberculin Skin Test in the Diagnosis of Bovine Tuberculosis

Determination of the Cut-Off Value of the Prepared ELISA:

Heparinized blood samples were collected from apparently healthy cows. These cows were then examined with the tuberculin skin test through the authorized personnel in the Ministry of agriculture using PPD (Tuberculin section, Institute of Vaccine and Serum Production, Ministry of Agriculture) and the result were recorded. The heparinized blood samples collected from 10 cows that proved to be tuberculin negative were examined in vitro for IFN-γ production according to (Wood and Jones, 2001; Smith et al. 2021). A briefly sterile 0.1mL PPD solution was added to a 10mL blood sample collected from each animal under a completely sterile condition in heparinized screw-capped tubes. The blood samples were incubated at 37ºC and 5% CO2 for 16 hrs. Then the tubes were centrifuged at 1000xg for 10 min. the plasma was collected and examined with the prepared ELISA test for its content from IFN-γ and the ELISA COV was determined.

Measurement of the Bovine IFN-γ using the Developed ELISA

Using the prepared ELISA plates coated with the anti-Bo-IFN-γ specific mAbs, the tested plasma samples were distributed in 100µL/well. The plates were incubated at 37ºC for 1 hour, washed 3 times with PBS-tween 20 (0.05%) washing buffer, and dried. After that 100µL/well peroxidase conjugated anti-Bo-IFN-γ mAbs 1/1000 dilution (kindly supplied from Harbin-Weike Research Institute, China) was added to each well. The plates were incubated for 1hr at 37ºC and then washed 3 times using the washing buffer. The plates were dried and 100/well ABTS substrate solution (Peroxidase SubstrateOABTS (1 Component) SIGMA-ALDRICH) was added, and the plates were further incubated at 37ºC for 30 min. A stopping solution (1M H_2SO_4) was added to all wells (100µL/well) and the OD was determined using a spectrophotometer.

Determination of the Sensitivity and Specificity of the Bo-IFN-γ ELISA

In a preliminary study heparinized blood samples were collected from 23 apparently healthy cattle and tested for IFN-γ production using the prepared ELISA. These animals were tested by the tuberculin skin test and the results were recorded. The obtained results of the Bo-IFN-γ ELISA test were compared with the results of the tuberculin skin test and the sensitivity and specificity of the Bo-IFN-γ prepared ELISA test was determined. All samples involved and provided through this step were obtained after permission was taken from the owner of the cows.

RESULTS

Production of Murine Hybridoma Producing Bo-IFN-γ Specific Monoclonal Antibodies

The fusion of splenocytes of Bo-IFN-γ immunized BALB/C mice with P3NS1 myeloma cells yielded a total of 54 HAT-resistant hybridoma clones, 19 clones of secreted mAbs reacting specifically with Bo-IFN-γ antigen (Table 1). The Bo-IFN-γ monoclonal antibodies secreted by these clones were of IgM isotype (Fig. 1). The specificity of the produced mAbs was proved using the western blotting technique. Out of the 19 positive hybrids only three were successfully cloned, namely, 1A1, 2D2, and 3H1. These Clones were then expanded, cloned, and stored in liquid nitrogen.

Determination of the Cut-off Value of the Prepared Bovine IFN-γ ELISA

As shown in (Table 2), the OD reading of Bo-IFN-γ ELISA testing of blood from 10 apparently healthy tuberculin negative cattle were recorded and the cut-off value was determined and found to be equal to 0.30.

Table 1: Results of screening of the fusion plates, 12 days post fusion, for hybridomas producing anti- Bo-IFN-γ monoclonal antibodies using ELISA

No. of Plates	No. of wells showing hybridoma	Positive wells No.	Positive wells %	Negative wells No.	Negative wells %
1	21	10	47.6	11	52.4
2	20	4	20.0	16	80.0
3	13	5	38.5	8	61.5
Total	54	19	35.4	35	64.6

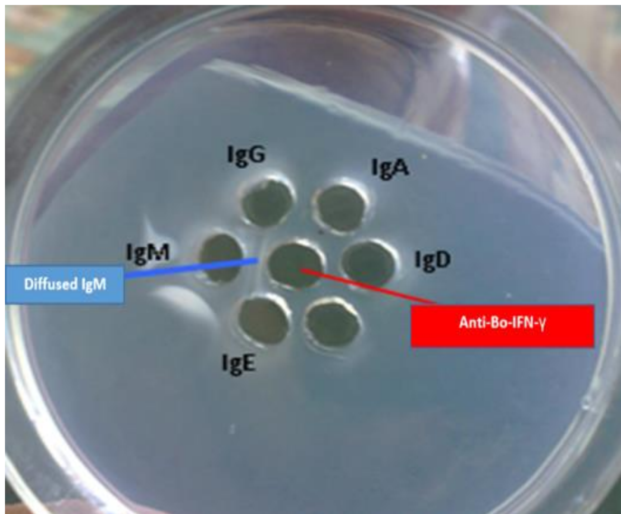

Fig. 1: The produced Bo-IFN-γ specific mAbs of IgM isotype.

Sensitivity and Specificity of the Prepared ELISA in the Diagnosis of Bovine Tuberculosis as Compared to the Tuberculin Skin Test

A total of 23 cows were examined by the developed bovine IFN-γ ELISA test and by the standard tuberculin skin test, which was done through the general organization for the veterinary services. As shown in (Table 3), five cows (Numbers: 19 - 23) were positive in the tuberculin skin test and in the Bo-IFN-γ ELISA test showing OD readings equal to 0.78, 0.38, 0.65, 0.34, and 0.78, respectively. Fourteen cows (1, 2, 3, 6-10) were negative in both tests where the ELISA COV value reading was below 0.30. Two tuberculin-negative cows (No. 4 and 5) gave false positives using the prepared Bo-IFN-γ ELISA showing OD equal to 0.31 and 0.89, respectively. Also, two tuberculin-positive cows (No. 17 and 18) were false negative in the ELISA and showed COV below 0.30. It was recorded by (Ferrara et al. 2009) that delay of examination of collected samples above 6 hours results in false positive results. The calculated sensitivity and specificity in this preliminary study were 98% and 71.4%, respectively.

DISCUSSION

Diagnostics based on conventional testing are insufficient, particularly for intracellular infections. Therefore, better immunodiagnostic techniques are required to identify cellular immunity. Because of its greater sensitivity, repeatability, accessibility, and adaptability to various antigens, in vitro applications are preferred to in vivo experiments. Additionally, they offer superior logistical support and shorter test intervals (Rothel et al. 1992; De la Rua-Domenech et al 2006; Gormley et al 2006). The creation of in vitro tests to quantify T cell Bo-IFN-γ release, which has competed and replaced the tuberculin skin test, has been a significant advancement (Rothel et al. 1992; Dockrell and Weir 1998; Jungersen et al. 2002; Gul et al. 2022).

With a sensitivity of 95.2%, the Bo-IFN-γ test has been used in conjunction with the tuberculin skin test in eradication operations to diagnose bovine tuberculosis (Rhodes et al. 2000). Additionally, it was shown that the test can recognize those actively infected yet skin test-negative animals (Neil et al 1994). Therefore, the current work goal was to create a Bo-IFN-γ ELISA based on murine monoclonal antibody reagents for the quantitative measurement of the Bo-IFN-γ response in samples of bovine heparinized blood samples. Utilizing verified plasma samples, the accuracy and test performance were assessed for this purpose. Only plasma samples with bovine PPD positivity that were verified by tuberculin test were utilized in this investigation to evaluate the Bo-IFN-γ level.

So, the Bo-IFN-γ content of the control negative and positive plasma samples as well as the 5 positive, 14 negative, and the 4 un-concordant plasma samples as detected by tuberculin test and Bo-IFN-γ ELISA were compared. The findings demonstrated that PPD antigens can be used to detect Bo-IFN-γ in plasma at different concentrations. The prototype could clearly identify Bo-IFN-γ in blocking solutions, plasma, and sera when the test was evaluated with various concentrations and dilutions of Bo-IFN-γ.

Table 2: Determination of the Cut-off value (COV) of the prepared Bo-IFN-γ ELISA for diagnosis of bovine tuberculosis.

Cow No.	Result of Tuberculin test* First reading	Second reading	Difference	Interpretation**	ELISA Bo-IFN-γ level expressed as OD
6557	10	10.8	0.8	Negative	0.28
6643	11	11.9	0.9	Negative	0.22
6691	10	10.8	0.8	Negative	0.15
6769	11	11.9	0.9	Negative	0.16
6842	9	9.9	0.9	Negative	0.18
6899	10	11	1.0	Negative	0.17
7001	11	11.9	9.9	Negative	0.22
7027	11	12	1.0	Negative	0.28
7038	10	11.1	1.1	Negative	0.19
7050	11	11.9	0.9	Negative	0.24

*The cows were examined by tuberculin test through the authorized personnel in the Veterinary Service Organization-Ministry of Agriculture and proved to be tuberculosis negative by the tuberculin skin test. **ELISA reading below 0.30 is considered negative.

Table 3: Comparison between the results of the tuberculin skin test and the Bo-IFN-γ ELISA test

		Diagnosis by Tuberculin test			Diagnosis by BO-IFN-γ ELISA test	
Serial No.	Animal No.	Skin thickness (mm)	Difference	Diagnosis	OD*	Diagnosis
1	7069	10	10.8	0.8	Negative	0.18
2	7081	11	12	1.0	Negative	0.18
3	7085	10	11.1`	1.1	Negative	0.11
4	7144	11	11.9	0.9	Negative	0.31
5	7164	10	11	1.1	Negative	0.89
6	7206	11	11.8	0.8	Negative	0.11
7	7212	10	10.9	0.9	Negative	0.18
8	7269	11	12	1.0	Negative	0.19
9	7272	10	11.0	1.1	Negative	0.19
10	7292	11	11.9	0.9	Negative	0.11
11	7301	10	11.0	1.0	Negative	0.22
12	7352	11	11.8	0.8	Negative	0.19
13	7356	10	10.9	0.9	Negative	0.12
14	7362	11	11.8	0.8	Negative	0.26
15	7388	10	10.8	0.8	Negative	0.28
16	7397	11	11.9	0.9	Negative	0.19
17	710	6	17	11	Positive	0.13
18	708	10	15	5	Positive	0.18
19	801	25	31	6	Positive	0.78
20	721	16	26	10	Positive	0.38
21	811	21	30	9	Positive	0.65
22	733	22	30	8	Positive	0.34
23	791	17	25	8	Positive	0.73

In this work, clone 1A1 monoclonal antibody-derived primary antibody results for Bo-IFN-γ ELISA were shown to be more specific than clone 2D2 and 3H1 but less sensitive and accurate.

As a result, the shelf life of a prototype made with main Ab from clone I was determined. Although the results are satisfactory, a new evaluation of this prototype using more samples that characterize tuberculosis status is still required.

In order to diagnose *M. bovis* in cattle, antigen-specific activation of Bo-IFN-γ production was used as a diagnostic tool (Wood et al. 1990; Neil et al. 1994). To test the effectiveness of the developed Bo-IFN-γ assay as a detection tool and to learn more about the Bo-IFN-γ quantity of the evaluated plasma samples that were stimulated with bovine PPD antigens, we designed the current study. Although the detection limit of quantitative ELISA detecting Bo-IFN-γ concentrations is approximately 50-2000pg/mL, there is no accurate information available on Bo-IFN-γ levels in bovine TB-infected animals.

The evaluation of the test findings can be done in accordance with the difference or the fraction of OD values of Bo-IFN-γ in plasma samples induced with a certain protein antigen, as the animals sensitized with the agent can be detected within that range. However, some researchers take into account Bo-IFN-γ levels as opposed to OD value interpretation.

Mikkelsen et al. confirmed positive in PPD-stimulated samples with Bo-IFN-γ concentration ≥1 ng/mL when interpreting the results (Mikkelsen et al. 2009). In our study, confirmed plasma samples stimulated with bovine PPD were evaluated and the results were categorized at a range of 0.11-0.78 OD. This classification, whether depending on concentration detection or on OD value interpretation, is not strict and with testing more samples wider range may be figured out. The current research emphasizes the necessity for tests that assess humoral immune response in addition to the reference standardized tuberculin skin test, such as the lateral flow immunochromatographic (LFIC) strips or ELISA (Alonso et al. 2021; Soliman et al. 2023). The newly developed detection method created in this study may help bTB management programs and strategies be improved by reducing the number of positive *Mycobacterium bovis*-infected cattle that remain on dairy farms as well as continuously shed and spread the disease.

Conclusion

With a worldwide problematic existence of tuberculosis, however, as a disease of poverty and insufficient immunity-related pre-requesting conditions, the main areas affected by such disease are those categorized as low-income, developing countries, so a proper easily to be produced, and distributed accurate test enforcing a national tuberculosis control strategy is a must. The current conducted study tried to achieve the previously highlighted insight by the production of murine monoclonal antibodies as well as developing a rapid and accurate ELISA for diagnosis of the main tuberculosis biomarker, interferon-gamma. The developed kit showed a very promising and competitive result in comparison to the regular authority-approved tuberculin skin test.

The obtained results indicate the possibility of using the developed Bo-IFN-γ ELISA kit as a simple, rapid, accurate, and sensitive test that can be applied to a large number of animals in a herd or even on a national tuberculosis screening scale, identification, and eradication of tuberculosis-positive cases, helping in the overall national tuberculosis control strategies.

Availability of Data and Material (ADM)

The data used and/or analyzed related to the animal cases tested during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

Authors' Contributions

All authors contributed to the article and approved the submitted version. All authors contributed through different stages of the study as follows; Rafik Soliman and Hassan Aboul-Ella were involved in the study conceptualization of the methodology, Yehia Badr, Mona Abd El Aziz, and Zeinab Hashem were involved in the validation, investigation, and analysis of the obtained results, Rafik Soliman and Hassan Aboul-Ella were responsible for primary draft writing, final writing, review, and editing.

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