Potential Interference of Intradermal Tuberculin Testing in Serological Diagnosis of Bovine Tuberculosis

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Article

Potential Interference of Intradermal Tuberculin Testing in Serological Diagnosis of Bovine Tuberculosis

Yi-Ying Chen [1,2], Chih-Yu Wen [1], Shin-Wu Liu [1], Wen-Chin Lin [2,3], Jacky Peng-Wen Chan [1] and Chih-Jung Kuo [1,*]

[1] Department of Veterinary Medicine, College of Veterinary Medicine, National Chung Hsing University, Taichung 40227, Taiwan

[2] Institute of Preventive Medicine, National Defense Medical University, New Taipei City 23742, Taiwan

[3] Graduate Institute of Pathology and Parasitology, National Defense Medical University, Taipei 11490, Taiwan

* Correspondence: ck476@nchu.edu.tw

Simple Summary

Bovine tuberculosis (bTB) is an infectious disease of cattle that can also affect humans. The intradermal tuberculin test (ITT) is commonly used worldwide to screen cattle for bTB. Meanwhile, antibody-based blood tests (enzyme-linked immunosorbent assays [ELISAs]) are increasingly applied as complementary diagnostic tools. However, the interaction between these two testing methods may influence diagnostic accuracy. Our findings indicate that ITT can temporarily stimulate antibody responses against *Mycobacterium bovis*, leading to false-positive ELISA results in bTB-free cattle. To reduce the risk of misdiagnosis, we recommend avoiding serum collection for bTB antibody testing for at least 21 weeks following ITT administration.

Abstract

A zoonotic disease caused primarily by *Mycobacterium bovis* (*M. bovis*), bovine tuberculosis (bTB), remains a considerable global concern. The intradermal tuberculin test (ITT) is a primary global screening tool for infected animals through their cellular immune response. However, ITT fails to identify all bTB-infected animals. Serological enzyme-linked immunosorbent assays (ELISAs), which detect humoral immune responses are a potential complementary approach for bTB diagnosis. Herein, 86 serum samples collected from a bTB-free herd were analyzed using three bTB serological ELISA kits: the IDEXX *M. bovis* antibody test (IDEXX), the BIONOTE BTB antibody ELISA 2.0 kit (BTB), and an in-house ELISA using MPB70 and MPB83 as antigens (termed homemade). Antibody responses were monitored before and after ITT administration for 21 weeks. All serum samples collected before ITT administration tested negative with all three ELISA kits. However, 1 week after ITT administration, samples tested positive using the IDEXX, BTB, and homemade ELISA kits. Week 9, all samples tested negative with the BTB and homemade ELISA kits, whereas for IDEXX they remained negative until week 21. ITT-induced a serological response against *M. bovis*, engendering false-positive results. Therefore, collecting serum samples for bTB antibody testing should be avoided for at least 21 weeks following ITT.

Keywords: bovine tuberculosis; *Mycobacterium bovis*; intradermal tuberculin test; serological ELISA; diagnostic interference; false-positive reaction; MPB70; MPB83

1. Introduction

Bovine tuberculosis (bTB) is a contagious disease that is caused by *Mycobacterium bovis*, affecting both animals and humans, leading to substantial economic losses in many countries [1]. *M. bovis*

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spreads primarily through respiratory secretions from infected animals, direct contact with contaminated materials, or consumption of contaminated dairy products. Controlling and eliminating bTB remains challenging, mainly due to the high cost of surveillance methods and limited diagnostic test sensitivity. The design and implementation of bTB surveillance systems require the ability to detect infections in cattle as early as possible to minimize transmission and reduce the costs associated with control and eradication efforts [2].

Following infection, macrophages initiate a range of antibacterial mechanisms in response to *M. bovis* [3]. However, the bacterium can survive and evade many of these responses. This leads to the development of active TB or reactivation from dormancy at a later stage [4]. *M. bovis* can induce a delayed-type hypersensitivity (DTH) reaction mediated by T lymphocytes [4–6]. The DTH response is an indicator of cellular immune activation resulting from infection and disease caused by *Mycobacterium* spp. [7]. The role of B-cell responses has been demonstrated, suggesting that humoral antibody production is only detectable in the advanced stages of bTB. This indicates a shift from a predominantly cellular immune response to a humoral immune response in bTB-infected animals [3,4].

bTB diagnosis in domestic ruminants primarily relies on the intradermal tuberculin test (ITT), comparative intradermal tuberculin test (CITT), and interferon-gamma (IFN-γ) release assay [8], all of which are based on cellular immune responses. The ITT, a standard method recommended by the World Organization for Animal Health has been widely used as a diagnostic strategy in animals to measure delayed hypersensitivity responses to purified protein derivative (PPD) prepared from *M. bovis* [9]. After 48–72 h, a healthcare professional examines the injection site for a reaction. ITT is an essential tool in bTB control and prevention programs, aiding in the identification of infected animals that may require further evaluation or treatment for latent bTB infection. This can help to curb the spread of bTB within communities [9]. The CITT involves the intradermal injection of avian and bovine PPDs, derived from *M. avium* complex and *M. bovis*, respectively. This test helps differentiate *M. bovis* infection from infections caused by nontuberculous mycobacteria [10]. The IFN-γ release assay detects cell-mediated immune responses to *M. bovis* by measuring the production of IFN-γ from white blood cells (lymphocytes) stimulated with specific *M. bovis* antigens. This assay can identify some tuberculin skin test-negative cattle, as it can detect infections in very early stages [11,12]. It is generally used in conjunction with other diagnostic tests to improve the accuracy of bTB detection in cattle herds. However, as the disease progresses, the cellular immune response shifts to a humoral response. Therefore, traditional cellular immunity-based diagnostic methods may fail to identify all bTB-infected animals [13]. The humoral immune response is characterized by increased IgG1 antibodies against *M. bovis* antigens [4,13], which can be detected using immunoassays, such as enzyme-linked immunosorbent assays (ELISA).

The serological diagnosis of bTB using ELISA is a widely used method in veterinary medicine to detect specific antibodies produced by the humoral immune response against *M. bovis*. ELISA is a highly sensitive and specific laboratory technique designed to identify antibodies or antigens in biological samples. In the case of bTB, ELISA is used to detect antibodies in serum, plasma, or milk samples from cattle suspected of being infected with *M. bovis*. Various antigens derived from *M. bovis*, such as antigen 85 (Ag85) complex [14], Mycobacterial Protein B83 (MPB83) [15], Early Secretory Antigenic Target 6 (ESAT-6) [16], Culture Filtrate Protein 10 (CFP-10) [17,18], MPB70 [19], and the recently developed P22 protein complex [20], can be used in bovine TB ELISA to capture specific antibodies present in the tested samples. Several commercial serological ELISA kits for bTB diagnosis, such as the IDvet ELISA, IDEXX Laboratories ELISA, and BioNote BTB Ab ELISA 2.0 Test Kit, are currently available. When combined with cellular-based tests, serological ELISA is a valuable diagnostic tool for monitoring bTB at the herd level. Its effectiveness in maximizing the identification of TB-infected ruminants has been well-demonstrated. In Taiwan, annual ITT has been used as an official testing for diagnosing bTB in cattle, dairy goats, and domestic deer. Meanwhile, serological ELISA is a companion diagnostic test to enhance diagnostic accuracy of bTB. However, the potential interference ITT in bTB serological ELISA has not been clarified. Herein, the effects of ITT on

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serological ELISA were investigated and appropriate timing of blood sampling for ELISA was suggested [8,21,22].

2. Materials and Methods

2.1. Study Design and Sampling Collection

Eighty-six Holstein dairy cows were selected from a bTB-free farm affiliated with National Chung Hsing University in Taiwan. The herd had remained negative for annual ITT since 2015. All the cows were housed, fed, and monitored as previously described [23]. Blood samples were collected on the day of ITT administration (week 0, before injection) and at 1, 2, 4, 9, 14, 17, and 21 weeks following ITT (Figure 1a). To reconfirm the bTB-free status of the herd, ITT was performed again at week 26. Serum samples were obtained by centrifugation of blood at 3000 × g for 10 min for the ELISA analysis.

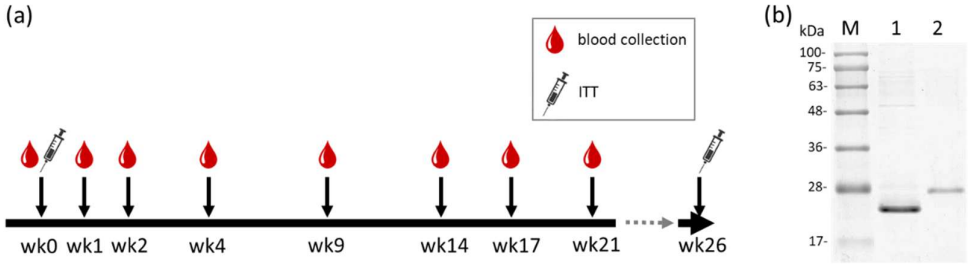

Figure 1. Experimental design and antigen characterization. (a) Schematic overview of the experimental timeline. Blood samples were collected before the intradermal tuberculin test (ITT, week 0) and at weeks 1, 2, 4, 9, 14, 17, and 21. The ITT was repeated at week 26 to confirm the bTB-free status of selected cows. (b) Preparation and characterization of MPB70 and MPB83. SDS-PAGE analysis of purified recombinant MPB70 (24.6 kDa, lane 1) and MPB83 (28.6 kDa, lane 2). Lane M represents molecular mass markers (100, 75, 63, 48, 35, 28, and 17 kDa).

2.2. ITT Analysis

The ITT was conducted by government veterinarians from a different county. An intradermal injection of 0.1 mL of PPD-B tuberculin (25,000 IU/mL; CZ Vaccines, Pontevedra, Spain) was administered to the caudal fold of the cows. Visual examination and palpation were conducted 72 ± 6 h after injection. Swelling or other clinical signs at the injection site were considered positive reactions.

2.3. Preparation of M. bovis Antigens

Two major antigens of *M. bovis*, namely, MPB70 (Mb2900) and MPB83 (Mb2898), were selected as antigens for the homemade ELISA kit. The genes encoding MPB70 and MPB83 were amplified from the *M. bovis* (ATCC19210) genomic DNA and cloned into the pET16b vector using PCR with the forward primer 5′-ggaattccatatgaaacccgtgtcgcaagacac- 3′ containing NdeI cleavage sequences; reverse primer 5′-ccgctcgagttactgtgccgggggcatc- 3′ containing XhoI cleavage sequences; 5′-ggaattccatatgggcgatctggtgggcc - 3′ containing NdeI cleavage sequences; reverse primer 5′-ccgctcgagttactgtgccgggggcatc- 3′ containing XhoI cleavage sequences; 5′-ggaattccatatgggcgatctggtgggcc - 3′ containing NdeI cleavage sequences; and reverse primer 5′-ccgctcgagttacgccggaggcattagc- 3 containing XhoI cleavage sequences for MPB70 and MPB83, respectively. The recombinant MPB70 or MPB83 plasmid was then used to transform the *Escherichia coli* DH5alpha competent cells. The transformed cells were streaked on a Luria–Bertani (LB) agar plate containing 100 mg/mL ampicillin. Ampicillin-resistant colonies were selected from the agar plate and sequenced to detect the target genes. The correct construct was subsequently transformed to *E. coli* BL21 (DE3) for protein expression. The 5-mL overnight culture of a single transformant was

 doi:10.20944/preprints202604.0663.v1

used to inoculate 500 mL of fresh LB medium containing 100 mg/mL ampicillin. The cells were grown to an optical density at 600 nm of 0.6 and induced with 0.5 mM isopropyl-β-thiogalactopyranoside. After 4–5 h, the cells were harvested by centrifugation at 7,000 × g for 15 min. The MPB70 or MPB83 was conducted at 4 °C. The cell paste obtained from 1-L cell culture was suspended in 40 mL lysis buffer containing 25 mM Tris-HCl (pH 7.5) and 150 mM NaCl. A French press instrument (AIM-AMINCO spectronic instruments; Cambridge Scientific Products, MA, USA) was used to disrupt the cells at 12,000 lb/in2. The lysis solution was centrifuged, and the debris was discarded. The cell extract was loaded onto a 5-mL Ni-NTA column equilibrated with the same buffer containing 5 mM imidazole. The column was washed with 5 mM imidazole followed by 30 mM imidazole-containing buffer. The His-tagged MPB70 or MPB83 was eluted with the lysis buffer containing 300 mM imidazole. The protein solution was dialyzed against 1 L of lysis buffer two times. The enzyme concentrations used in experiments were determined based on the absorbance at 280 nm and purity of recombinant protein was evaluated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. IDEXX M. bovis Ab Test Procedure

The IDEXX *M. bovis* Ab test, termed IDEXX ELISA, was used in accordance with the manufacturer's instructions (IDEXX Laboratories, Westbrook, ME, USA). Serum samples and controls diluted at a 1:50 ratio were dispensed into the wells and incubated for 60 min at 18–26 °C. After a washing step, the conjugate was added and plate was incubated for 30 min at 18–26 °C. The process was performed by adding chromogen substrate. The reaction was stopped using Stop Solution after 15 min at 18–26 °C. The ELISA test results were expressed as the value of the sample (S) divided by the value of the positive control serum (P) supplied in the IDEXX ELISA kit, as determined by measurement of the optical density (OD460) with an ELISA plate reader (Multiskan GO, ThermoFisher). The reading obtained from each sample divided by the value of the positive control was used to calculate the sample/positive (S/P) value for each sample. A positive result was defined as an S/P ratio ≥ 0.30 and a negative result was defined as an S/P ratio < 0.30.

2.5. BIONOTE BTB Ab ELISA Test Procedure

For the BIONOTE BTB Ab ELISA 2.0 Kit, termed BTB ELISA, the antibody response was examined following the manufacturer's instructions. The Linked Immunosorbent Assay for the qualitative detection of *M. bovis* antibody in serum. The BIO NOTE BTB Ab ELISA 2.0 kit contains a microplate, which is pre-coated with purified bTB antigen on the wells. The procedure recommended by the manufacturer was followed for the detection of antibodies against *M. bovis*. The S/P value was obtained according to the formula: S/P = [(OD460 sample-mean OD460 NC)/(mean OD460 PC-mean OD460 NC)] (PC: positive control; NC: negative control).

2.6. MPB70/MPB83 ELISA Test Procedure

ELISA using MPB70/MPB83 as capture antigens was performed using recombinant MPB70 and MPB83. In brief, purified MPB70 and MPB83 were mixed in a 1:1 weight ratio and coated on the ELISA plate (500 ng/well) using 0.1 M $NaHCO_3$ (pH 9.5) and incubated at 4 °C overnight. Unbound excess protein was removed by washing the wells twice with PBST containing 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, and 0.05% Tween 20 at pH 7.4. The wells were subsequently blocked with 3% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) at 37 °C for 1 h. The bovine serum sample was diluted at 1:1 in PBS plus 1% (w/v) BSA and loaded onto the ELISA duplicate wells following by incubation at 37 °C for 1 h. The wells were washed three times with 200 µL of 1 × PBST followed by incubation with 100 µL of rabbit anti-bovine IgG-HPR from goat (Jackson ImmunoResearch Inc, USA) diluted 1:50000 in PBS plus 1% (w/v) BSA. The wells were washed three times with 200 µL of 1 × PBST. The HPR substrate TMB (GBI Labs, USA) was added to the wells (100 µL). The reaction was stopped after a 10-min incubation at room temperature by adding 1 M HCl

 doi:10.20944/preprints202604.0663.v1

(100 µL/well). The OD_{460} was measured using an ELISA plate reader (Multiskan GO, ThermoFisher). The cut-off value was calculated 3× mean values at OD_{460} of negative control. Values ≥ 3× cut-off were considered positive while values under 3× cut-off were negative.

3. Results

3.1. Purification of MPB70 and MPB83

MPB70 and MPB83 were expressed with His-tags to facilitate purification using Ni-NTA affinity chromatography. The purity of each protein was confirmed to be > 90% by reducing SDS-PAGE. The estimated molecular weights of His-tagged MPB70 and MPB83 are 24.6 and 27.8 kDa, respectively (Figure 1b).

3.2. ITT Results

The ITT were administered at weeks 0 and 26. Visual examination and palpation were performed 72 ± 6 h after injection. None of the 86 cows showed substantial swelling or other clinical signs at the injection sites (data not shown). These results confirmed that all the animals were bTB-free.

3.3. IDEXX M. bovis Ab Test Results

As shown in Figure 1(b), all 86 serum samples tested negative using the IDEXX *M. bovis* Ab Test kit on the day of ITT administration (week 0). However, four samples (no. 15, 16, 32, and 86) tested positive at 1 week post-ITT, with S/P values of 5.9, 4.3, 2.5, and 3.3, respectively (Figure 2a). These four samples remained positive at weeks 2 and 4 but turned negative at weeks 9, 14, 17, and 21. Serum sample 14 tested positive at weeks 9, 14, and 17, with lower S/P values of 0.6, 0.5, and 0.3, respectively, but was negative at week 21. Samples 79 and 81 tested positive only at week 14 (S/P = 1.0) and week 4 (S/P = 0.3), respectively. The total number of positive samples was 0, 4, 4, 5, 1, 2, 1, and 0 at weeks 0, 1, 2, 4, 9, 14, 17, and 21, respectively (Figure 2b).

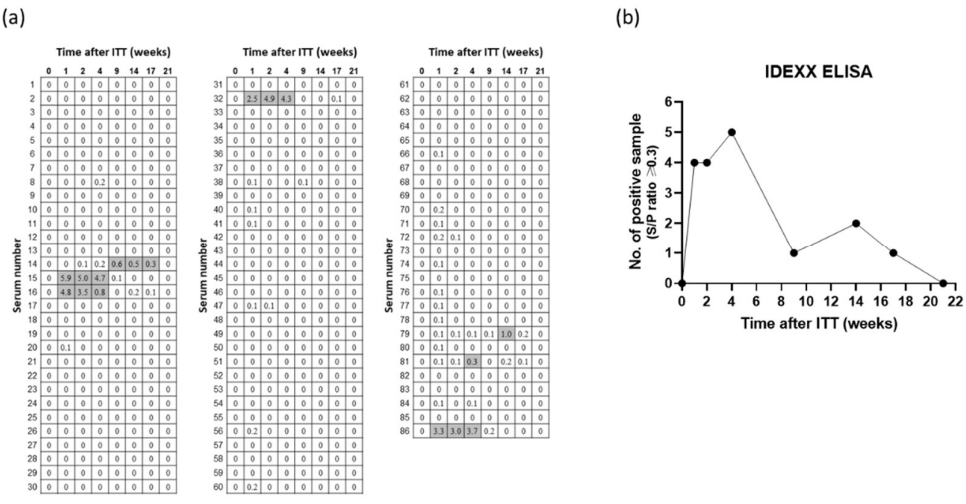

Figure 2. ELISA test results from 86 bovine sera blood samples using IDEXX ELISA kit. (a) Results are presented as the number of serum samples and their sample-to-positive (S/P) ratio values measured before and at 1, 2, 4, 9, 14, 17, and 21 weeks after the intradermal tuberculin test (ITT). An S/P ratio ≥ 0.3 was defined as positive and is indicated by gray boxes. (b) Number of positive antibody responses detected by the IDEXX ELISA in 86 *M. bovis*-free cows, recorded before and at 1, 2, 4, 9, 14, 17, and 21 weeks post-ITT.

3.4. BTB Ab ELISA Test Results

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Using the BTB Ab ELISA test kit, all 86 serum samples tested negative at week 0. At week 1, 12 samples, that is, nos. 8, 11, 15, 16, 25, 31, 32, 50, 53, 56, 59, and 86, tested positive. By week 2, samples 8, 11, 25, 31, 50, 53, 56, and 59 had reverted to negative, whereas samples 15, 16, 32, and 86 remained positive. Samples 20, 54, 60, and 61 tested positive at week 2. At week 4, only sample 16 remained positive. All the samples tested negative from weeks 9 to 21 (Figure 3a). The total numbers of positive samples were 0, 12, 8, 1, 0, 0, 0, and 0 at weeks 0, 1, 2, 4, 9, 14, 17, and 21, respectively (Figure 3b).

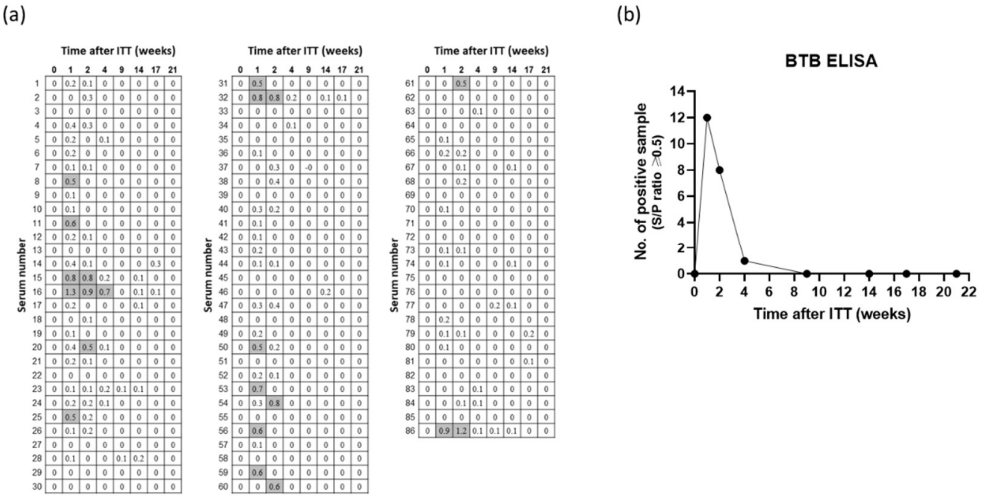

Figure 3. ELISA test results of 86 bovine sera blood samples using BTB Ab ELISA 2.0 ELISA kit. (a) Results are presented as the number of serum samples and their sample-to-positive (S/P) ratio values measured before and at 1, 2, 4, 9, 14, 17, and 21 weeks after the intradermal tuberculin test (ITT). An S/P ratio ≥ 0.5 was defined as positive and is indicated by gray boxes. (b) Number of positive antibody responses detected by the BTB Ab ELISA 2.0 in 86 *M. bovis*-free cows, recorded before and at 1, 2, 4, 9, 14, 17, and 21 weeks post-ITT.

3.5. MPB70/MPB83 ELISA Test Results

The mean OD_{450} value of bTB-negative serum, that is, the negative control, was 0.22, and the cut-off value was accordingly determined to be 0.66. Using the homemade ELISA kit with MPB70 and MPB83 as coating antigens, all 86 serum samples tested negative at week 0 (Figure 4a). At week 1, 4 samples, namely, no.: 15, 16, 32, and 86, tested positive. The number of positive samples increased to 6 at week 2, that is, no.: 15, 16, 20, 32, 47, and 86, and reached 7 at week 4 (no.: 15, 16, 20, 32, 47, 83, and 86). However, all the samples tested negative from weeks 9 to 21. The numbers of positive samples were 0, 4, 6, 7, 0, 0, 0, and 0 at weeks 0, 1, 2, 4, 9, 14, 17, and 21, respectively (Figure 4b).

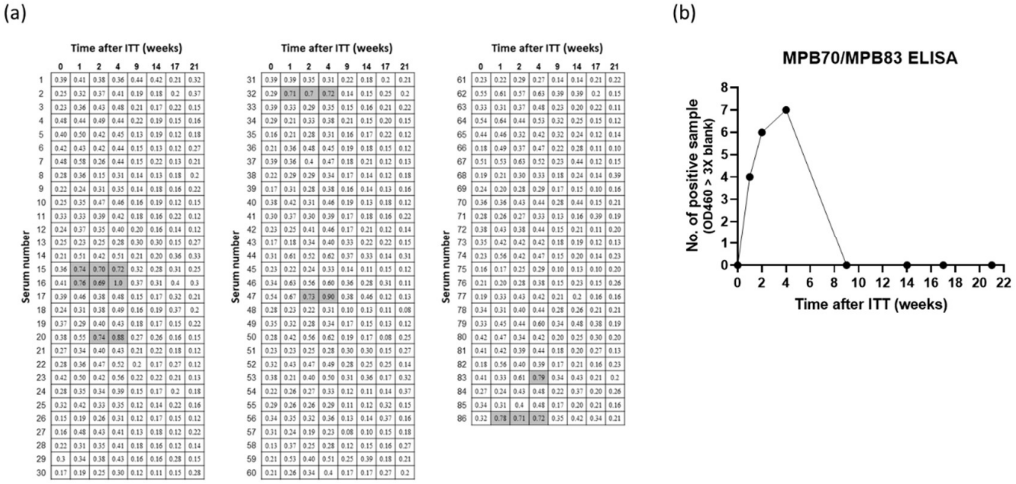

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Figure 4. ELISA test results of 86 bovine sera blood samples using MPB70 and MPB83 antigens. (a) Results are presented as the number of serum samples and their OD_{460} values measured before and at 1, 2, 4, 9, 14, 17, and 21 weeks after the intradermal tuberculin test (ITT). The cut-off value was determined to be 0.66. An OD_{460} value >0.66 was defined as positive and is indicated by gray boxes. (b) Number of positive antibody responses detected by the MPB70 and MPB83 antigens in 86 *M. bovis*-free cows, recorded before and at 1, 2, 4, 9, 14, 17, and 21 weeks post-ITT.

4. Discussion

bTB remains a considerable concern for animal and public health worldwide [24]. Several diagnostic methods are available for detecting *M. bovis* infection in cattle, which can be broadly classified as direct tests, for example, bacterial culture and PCR, and indirect tests, such as the single intradermal tuberculin test (SIT), CITT, IFN-γ assay, ELISA, and rapid lateral-flow tests. For direct detection, bacterial culture remains the standard for isolating *M. bovis* from postmortem samples of tuberculin-positive cattle. However, it requires several weeks for the results [25,26]. PCR provides a faster alternative, allowing detection of *M. bovis* DNA in clinical specimens such as blood, milk, and postmortem tissues [26]. Among the indirect tests available, SIT is the most widely used for early detection, measuring cell-mediated immune (CMI) responses that occur in the initial stages of infection [9,27]. Other CMI-based methods include the IFN-γ assay, which detects IFN-γ production following stimulation of blood lymphocytes with specific antigens [28,29]. Antibody-based tests such as ELISA and rapid lateral-flow assays are applied to detect *M. bovis* antibodies in blood or milk samples [30,31].

The tuberculin skin test is the recommended standard procedure for bTB ante-mortem diagnosis. It evaluates a delayed-type hypersensitivity reaction in sensitized cattle following an intradermal injection of purified protein derivative (PPD) tuberculin from mycobacteria, by measuring changes in skin thickness 72 h post-injection. Two commonly used intradermal tuberculin tests are the SIT and CITT. SIT uses bovine PPD (bPPD) but cannot distinguish between *M. bovis* infection and sensitization by environmental mycobacteria. In contrast, CITT administers both bovine and avian PPD simultaneously to improve specificity [10]. Despite its broad application, the tuberculin skin test exhibits a sensitivity range of 52–100%, averaging approximately 80% [32,33]. This implies that approximately 20% of infected cattle may remain undetected in a single round of testing, highlighting the need for supplementary diagnostic tools such as ELISA.

Serological ELISA is one of the options for detecting antibodies in bTB infected cattle using *M. bovis* antigens immobilized on the surface of microtiter plates. The purified antigens of *M. bovis*, such as the Ag85 complex [14], which comprises most secreted proteins, MPB70 [19] and MPB83 [15] proteins, which are highly homologous mycobacterial proteins with restricted distribution, are the antigens typically used to diagnose livestock infected with *M. bovis* [19]. MPB83 is a protein antigen found in *M. bovis* and is frequently used in bovine TB ELISA due to its high specificity for *M. bovis* antibodies [15]. ESAT-6 and CFP-10 are part of the mycobacterial ESX-1 secretion system and are specific to *M. tuberculosis* complex bacteria, including *M. bovis* [16–18]. They are also used in some TB ELISA assays. MPB70 [19] is a secreted antigen presented in *M. bovis* that can be used in bovine TB ELISA for detecting specific antibodies. P22 protein complex can be used in bovine TB ELISA to capture specific antibodies present in the tested samples [20]. Several commercial serological ELISA kits for bTB diagnosis, such as the IDvet ELISA, IDEXX Laboratories ELISA, and BioNote BTB Ab ELISA 2.0 Test Kit, are currently available. When combined with cellular-based tests, serological ELISA forms a valuable diagnostic tool for monitoring bTB at the herd level. Its effectiveness in maximizing the identification of TB-infected ruminants has been well-demonstrated.

bPPD inoculation for skin testing for TB increases the antibody responses to the *M. tuberculosis* complex in *M. bovis*-infected cattle [30,34,35]. Therefore, the serological tests for bTB in cattle should be performed after skin tests. Casal et al. [34] demonstrated that the use of serological testing performed following skin testing, in combination with traditional skin test procedures, increased the detection likelihood of TB animals within TB-infected cattle herds, as compared to skin tests alone. A

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study showed 63 animals from a herd free of both paratuberculosis, and TB were tested using the ITT [36]. Blood samples were collected before PPD inoculation and on days 3, 15, 30, 60, and 90 post-inoculation (p.i.). Sera were analyzed for paratuberculosis-specific antibodies using an ELISA based on protoplasmic antigen (ELISA-PPA), with results confirmed by a commercial ELISA. Between days 30 and 90 p.i., three animals (4.76%) tested positive by ELISA-PPA, and five animals (7.93%) tested positive from commercial ELISA. Thus, ITT may interfere with the accuracy of ELISA results. Serological testing for paratuberculosis should be avoided for up to 90 d following PPD administration (Figure 5).

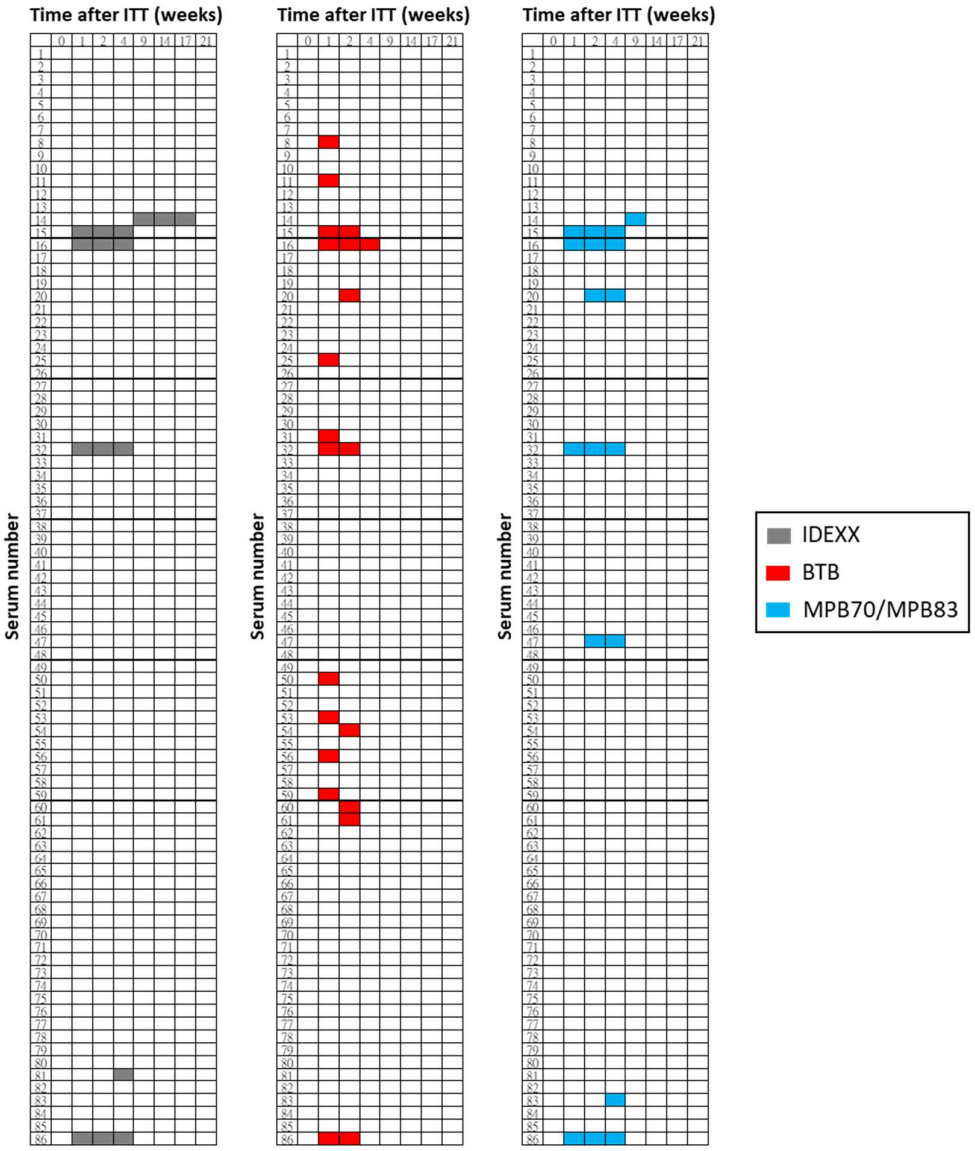

Figure 5. Comparison of ELISA results obtained with the IDEXX, BTB, and MPB70/MPB83 kits. Positive serum samples are indicated by gray, red, and blue boxes, respectively.

In Taiwan, annual ITT is used as the official test for diagnosing bTB in cattle, dairy goats, and domestic deer. Meanwhile, serological ELISA forms a companion diagnostic test to enhance accuracy. Our data demonstrates that intradermal tuberculin testing can temporarily alter the immune status of cattle, potentially leading to false-positive reactions in bTB ELISAs. To minimize this risk, it is

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recommended that blood samples for bTB serodiagnosis not be collected within 21 weeks following tuberculin testing.

5. Conclusions

Intradermal tuberculin testing can transiently alter the humoral immune response in cattle, leading to false-positive ELISA results even in TB-free herds. The interference is temporary but varies by assay, with IDEXX showing longer persistence than BTB and in-house ELISAs. To ensure diagnostic accuracy and avoid unnecessary interventions, serological testing for bTB should not be performed within 17–21 weeks of ITT. Adopting this interval will enhance the reliability of serological surveillance and support effective bTB management and eradication programs.

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Institutional Review Board Statement: All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (protocol number: 114008, approved on September 15, 2025). All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition) and the Animal Protection Act of Taiwan.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available on request from the authors.

Acknowledgments: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interests.

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