Colegio de Postgraduados

Comparison of a capture ELISA and Intradermal test for diagnosis of Bovine Tuberculosis in Mexico

Ramírez-Andoney, Vianey[1]; Alonso-Morales, Rogelio[2]; Gayosso-Vazquez, Amanda[2]; Pintor Ríos, Juan Pablo[2]; Escobar Chavarría Omar[1]; Acevedo-Jiménez, Gabriel Eduardo[1]; Guerrero-Chavez, Mayrem[2]; Flores-Huitrón, Nora Rosalia[1]; Díaz-Sánchez, Victor Manuel[2]; Autran-Martínez, Marcela[1]

[1] Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Cuautitlán, Facultad de Medicina Veterinaria y Zootecnia, México
[2] Laboratorio de Genética Molecular. Universidad Nacional Autónoma de México, Ciudad Universitaria, Av. Universidad #3000, Colonia, C.U., Coyoacán, 04510 Ciudad de México
* Correspondence: marcelaautranmartinez@cuautitlan.unam.mx

ABSTRACT

Objective: A comparative study was conducted to evaluate two immunodiagnostic techniques capture ELISA and the intradermal test for detecting bovine tuberculosis, aiming to establish the optimal test for early diagnosis in cattle.

Design/Methodology/Scope: Initially, the intradermal test was used as a screening tool in cattle, after which reactor animals underwent the comparative cervical test. Reactor animals (positive to the intradermal test) were then evaluated using a capture ELISA for bovine IFNγ. Intradermal testing is widely used in Mexico; however, its application presents several limitations. Identifying a faster and more accessible diagnostic test would significantly enhance disease control efficiency.

Results: The prevalence of bovine tuberculosis detected by the intradermal test was 4.9%, whereas the capture ELISA test identified 8.95%, showing a significant difference in the identification of positive cases between the two methods. The likelihood ratio met the standard parameters (LR+ >10 and LR− <0.5), confirming that the capture ELISA test for bIFNγis a valuable diagnostic tool. Additionally, the capture immunoassay detected a higher number of infected cattle compared to the intradermal test.

Conclusions/Limitations: The capture ELISA technique for bovine IFNγ proves to be a more effective diagnostic test. However, further studies are required, including a larger study population and expanded geographical coverage within the country.

Keywords: Bovine tuberculosis, Interferon gamma, Capture ELISA, Tuberculin skin test, sensitivity, specificity.

Citation: Ramírez-Andoney, V., Alonso-Morales, R., Gayosso-Vazquez, A., Pintor Ríos, J. P., Escobar-Chavarría, O., Acevedo-Jiménez, G. E., Guerrero-Chavez, M., Flores-Huitrón, N. R., Díaz-Sánchez, V. M., & Autran-Martínez, M. (2025). Comparison of a capture ELISA and Intradermal test for diagnosis of Bovine Tuberculosis in Mexico. *Agro Productividad*. https://doi.org/10.32854/agrop.v17i3.3279

Academic Editor: Jorge Cadena Iñiguez
Associate Editor: Dra. Lucero del Mar Ruiz Posadas
Guest Editor: Daniel Alejandro Cadena Zamudio

Received: October 18, 2024.
Accepted: March 16, 2025.
Published on-line: April XX, 2025.

Agro Productividad, 18(3). March. 2025. pp: 3-12.

INTRODUCTION

Bovine tuberculosis is a chronic infectious disease caused by *Mycobacterium bovis*, an acid-fast bacterium capable of infecting a wide range of hosts, including humans, making it a significant public health concern. In cattle, the disease leads to reduced body condition, decreased milk production, confiscation of affected organs, and quarantine

of infected animals, ultimately limiting livestock trade. These factors contribute to global economic losses exceeding $3 billion annually (Pérez-Morote, 2020). Bovine tuberculosis is present worldwide; however, its prevalence varies significantly between developed and developing countries. One of the primary factors influencing disease persistence is the ability to achieve timely diagnosis (Gormley, 2018; Lombard, 2021; Malama, 2013). Among the classical diagnostic methods, the intradermal tuberculin skin test is widely used. This test is based on detecting a type IV hypersensitivity immune response, requiring the inoculation of an antigen that stimulates memory lymphocytes, triggering a localized inflammatory reaction at the injection site, which becomes visible 72 hours post-inoculation (Carrisoza-Urbina J, 2019; Wangoo A, 2005). Currently, the most commonly used antigen for tuberculin testing is the purified protein derivative (PPD), obtained from secretion products of *M. bovis* strain AN5. The tuberculin testing protocol begins with a simple caudal fold test, in which only bovine PPD is administered. If the test is positive, a comparative cervical test must be performed to differentiate between reactions caused by *Mycobacterium avium* (NOM-031-ZOO-1995). There are also immunoassay-based diagnostic tests that detect key molecules involved in the immune response against *M. bovis*. These assays use blood samples and mycobacteria-derived antigens (such as PPD) to assess memory immune responses, primarily mediated by Th1 lymphocytes, which can be quantified through INF-γ detection (NOM-031-ZOO-1995; Ryan TJ, 2000). The first commercially available IFN-γ detection test for bovine tuberculosis was BOVIGAM® (Thermo Fisher, USA), which has been widely used in developed countries (Dean GS, 2005; Lahuerta-Marin A, 2015; Pollock JM, 2006; Praud A, 2015). However, high costs and lengthy import processes have driven the development of more affordable and accessible alternatives, particularly for developing countries, including various immunoassays that detect bovine INF-γ production, such as the one proposed in this study. Intradermal tests are widely used in Mexico; however, they present several practical limitations. These include the need for trained and certified personnel, as well as a minimum of 72 hours to obtain results. Consequently, the implementation of immunoassays could provide a faster and more accessible diagnostic approach for tuberculosis. In this study, the tuberculin test was compared against a specific capture ELISA for IFN-γ to evaluate the diagnostic potential of both methods in detecting bovine tuberculosis in cattle from various regions of Mexico.

MATERIAL AND METHODS

Ethical statement

This study was approved by the Institutional Committee for the Care and Use of Experimental Animals of the Universidad Nacional Autónoma de México, FES Cuautitlán (SICUAE-DC-2020/4-1), in compliance with the NOM-062-ZOO-1999 regulations.

Study animals

A random selection was conducted based on inclusion criteria, considering dairy, beef, and dual-purpose herds. The production system was batch-based and optimized according to the productive stage. Cattle were fed under a mixed system, consisting of

grains and coarse forages, with minimal concentrate supplementation and free access to water. A total of 384 cattle, aged 1 to 12 years, were included in the study, with their general characteristics detailed in Table 1. During the testing period and until the final determination of infection status, all cattle were kept in isolation. Animals confirmed as infected by any of the diagnostic tests were culled.

Intradermal test (Tuberculin skin test)

The two intradermal tests used in this study were performed by a licensed veterinarian. All animals included were pre-evaluated based on their clinical history. Cattle with records of vaccination, deworming, or tattooing within 30 days prior to the study were excluded.

Simple flow test

On day 0 of the experiment, the test was used as a general screening tool for the population. Proper livestock handling was ensured, and the skin fold thickness was measured at the center of the shaved area using a cutimeter. Subsequently, 0.1 mL of bovine PPD was intradermally inoculated into the caudal fold, with results interpreted 72 hours post-inoculation. Animals displaying swelling and/or other significant reactions were classified as reactor animals and were immediately subjected to the comparative cervical test.

Comparative Cervical Test

The comparative cervical test was performed 60 days after the caudal fold test. A total of 0.1 mL of avian PPD was inoculated into the upper middle third of the neck, while 0.1 mL of bovine PPD was administered in the lower neck area.

Bovine blood samples

Blood samples were collected in sodium heparin tubes (Heparin, BD®) via coccygeal and/or jugular venipuncture. Samples were transported at room temperature, protected from light exposure. A total of 250 μL of whole blood was placed in 96-well plates (Costar,

Table 1. General characteristics of the evaluated bovine study population.

Region	Number of bovines	*Bos indicus*	*Bos taurus*	Sex	
				Female	Male
State of Mexico (San Sebastián, Xhala, Cuautitlán Izcalli)	164	2	315	65	55
State of Mexico (San Sebastián, Teoloyucan)	62	8	13	25	30
Tabasco (Emiliano Zapata)	14	1	26	50	70
Querétaro (Colón, Querétaro Arteaga, Mexico)	144	2	13	69	20
Total	384	15	369	209	175

The different geographical regions of the animals were considered.

Corning). Cell stimulation was performed using 40 μg/well of bovine PPD (Pronabive, Mexico) for 24 hours at 37 °C. Plasma was then recovered by centrifugation at 3,000 rpm for 20 minutes, transferred into sterile tubes, and immediately evaluated.

Production of recombinant antigen (IFNγ)

The IFNγ gene was reamplified and cloned into the pET11a protein expression system (Novagen, Darmstadt, Germany) at the NdE1 and BamH1 sites, incorporating a 6x histidine tag at the carboxy terminus. Protein purification was carried out under native conditions using a Ni^{2+}-NTA affinity column (Qiagen, Chatsworth, CA, USA), following the manufacturer's instructions.

Obtaining monospecific polyclonal antibodies used in the capture ELISA technique

Specific antibodies against bIFNγ were generated in two species for use as primary and/or detection antibodies (anti-rabbit) and as secondary antibodies (anti-rabbit) for detection and quantification. The recombinant bovine IFNγ protein was utilized in the capture ELISA assay to measure bIFNγ production from bovine plasma.

Standardization of the capture ELISA technique for bovine IFNγ

The detection and quantification of anti-bovine IFNγ antibodies were performed using the IFNγ capture assay on serum samples from this study. The standardization of this technique and the production of the recombinant bovine IFNγ antigen were donated by the Laboratory of Molecular Genetics, UNAM, FMVZ.

Statistic analysis

The analysis included categorical data based on the positive or negative responses to both the intradermal test and the capture ELISA. An analysis of variance (ANOVA) with log-transformed data was performed to evaluate observed responses and conduct a comparative analysis between tests.

Statistical analysis was conducted using IBM® SPSS Statistics® Version 25 (IBM Corp.). Significant differences between diagnostic tests were determined using the McNemar test. The level of agreement between tests was assessed using Cohen's Kappa coefficient, while the likelihood ratio was also evaluated. Additionally, a comparative analysis of diagnostic tests was performed, measuring sensitivity (SE), specificity (SP), and positive predictive value (PPV).

RESULTS AND DISCUSSION

Intradermal test

All animals were evaluated using both intradermal test variants (CS and CC), with interpretation conducted by the same veterinarian. A total of 71 animals tested positive in the comparative cervical test, while 33 samples showed increased bovine IFNγ (bIFNγ) production in the capture ELISA test, a value compared against the test's negative controls. Approximately 3.1% of the animals were identified as positive in the comparative cervical

test, a finding consistent with previous reports by other authors (Perea-Razo, 2018; SENASICA, 2024).

Preliminary test to determine the sensitivity of the final bIFNγassay

Rabbit IgGs, at optimal dilutions of 4 μg and 8 μg, enabled clear discrimination between positive and negative samples. The minimum concentration of recombinant bIFNγ was 12 ng, while the maximum reached 200 ng. Serial double dilutions of bIFNγ were applied, as illustrated in Figure 1, showing IgG reactivity towards bIFNγ. These results confirm the proper functionality of the system. This assay allowed for the estimation of sensitivity values while also serving as a control curve within the system, providing both qualitative and quantitative data on bIFNγ detection in tuberculin-positive and negative animals.

Test with problem sera

Under the conditions established for the capture ELISA assays using a defined amount of rabbit IgG (4 μg) and guinea pig IgG (8 μg) bIFNγ was detectable within a range of 200 ng to 12 ng. The entire cattle population was evaluated, from which 22 serum samples were selected (12 positive and 10 negative for TB) based on reactivity to the tuberculin skin test (CC variant). Additionally, a positive control with varying concentrations of recombinant bIFNγ and a negative control (sample without IFNγ) were included, with each serum sample tested in duplicate. The assay results are presented in Figure 2, Population Evaluated Using Different Diagnostic Tests.

The commercial BOVIGAM test detects bIFNγ at a sensitivity of 80 pg/mL, which directly impacts the test's sensitivity when compared to the SE of this study (WOAH, Procedure for Registration of Diagnostic Kits, 2018).

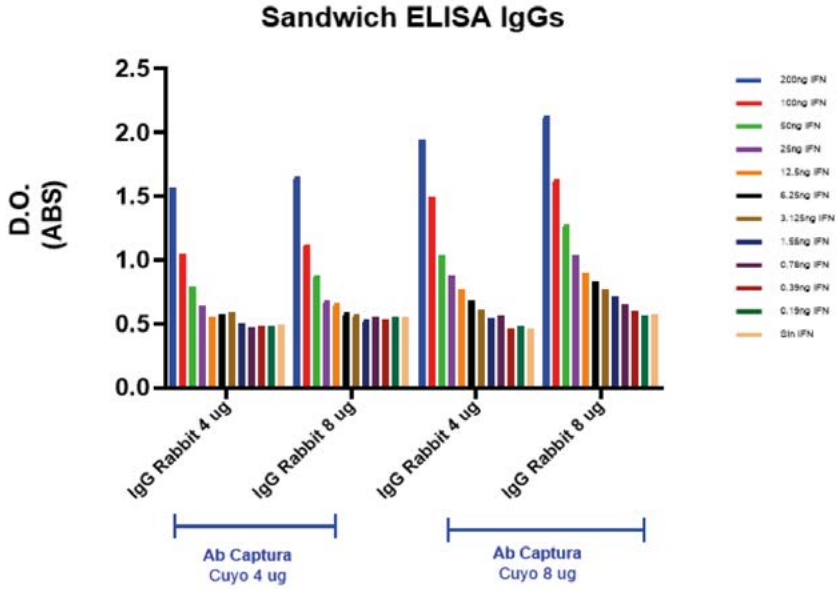

Figure 1. Reactivity of IgGs towards bovine gamma IFN. Sensitization of a rabbit and guinea pigs IgGs towards bovine IFNg, using monospecific polyclonal antibodies.

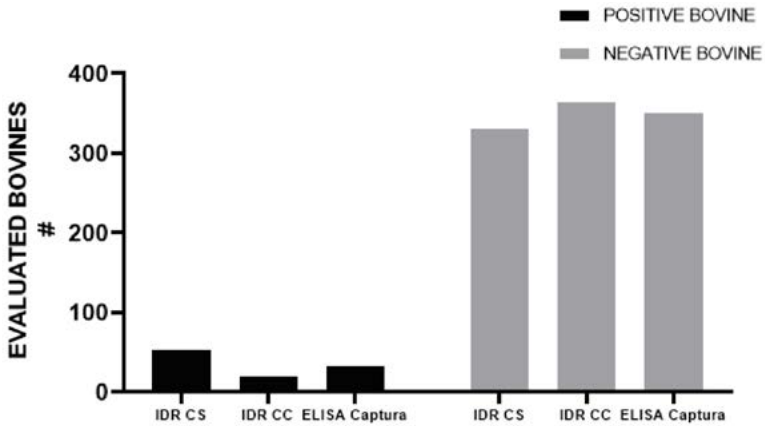

Figure 2. Total population evaluated towards different tests for the Diagnosis of Bovine Tuberculosis. Reactor animals ■ and animals negative ■ for 2 variants of tuberculinization are shown: simple caudal intradermorreaction (IDR-CS), cervical comparative reaction (DR-CC) and the capture ELISA test.

Capture ELISA Assay Standardization

The standardization of this assay enabled the evaluation of the capture ELISA test in comparison with the tuberculin skin test, identifying concordant elements between both methods and correlating the increase in bIFNγ levels in serum samples from bovines reacting to the comparative cervical test (Figure 3, Capture ELISA for the Quantification of bIFNγ). The rise in this cytokine is indicative of an active *Mycobacterium bovis* infection. Experimental and clinical data suggest that IFN-γ plays a crucial role in host defense against *Mycobacterium tuberculosis*. Deficiencies in IFN-γ production are considered a significant risk factor for *M. tuberculosis* infection and disease progression in humans (Dean GS, 2005).

Currently, global diagnostic strategies aimed at eradicating bovine tuberculosis have demonstrated that tests assessing cellular immunity such as this assay significantly reduce

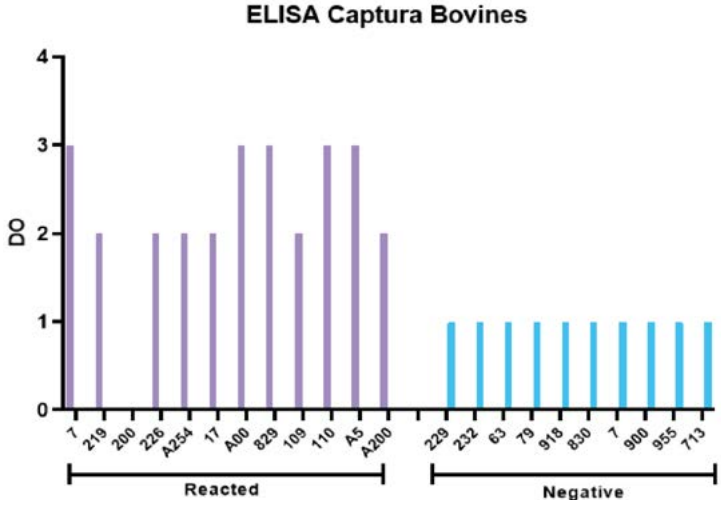

Figure 3. Capture ELISA based on the detection and quantification of bovine gamma IFN. The evaluation was carried out based on the selection of plasma from bovines that reacted to comparative cervical tuberculinization and from negative animals (n=12); The total number of samples is not included (n=384).

the presence of infected animals on farms (Alcaraz-López OA, 2016; Clegg TA, 2011; Clegg TA, 2017; Müller B, 2015).

Of the 384 cattle evaluated, 52 were identified as reactors to the intradermal screening test using the single caudal fold test. Among these, 19 animals tested positive in both the intradermal test and the capture ELISA assay. When comparing the effectiveness of both diagnostic methods in detecting positive animals, the intradermal test identified 4.94% of cases, whereas the capture ELISA for bIFNγ detected 8.95%. The discrepancy between the two tests may be attributed to factors such as sample handling during shipment, the time elapsed between collection and processing, and the low concentration of viable IFNγ in serum samples. According to WOAH (2018), the global prevalence of bovine tuberculosis varies across different geographic regions. In Mexico, data from SENASICA indicate that 86.27% of the national territory has reached the eradication phase, with a prevalence of less than 0.5% (SENASICA, 2024). However, in dairy cattle, an increase in prevalence of up to 16% has been reported (Perea-Razo, 2018).

The comparative analysis of diagnostic tests revealed that the capture ELISA for bIFNγ demonstrated 68% sensitivity, 95% specificity, a positive predictive value of 42%, a negative predictive value of 98%, and an overall prevalence of 0.05%. In contrast, commercial capture ELISA assays utilizing monoclonal antibodies, such as BOVIGAM, have reported a sensitivity range of 84-93% and specificity between 94-100% (Wood, 2001). The lower sensitivity observed in this study may be associated with the use of polyclonal antibodies in the ELISA assay; however, the test maintains high specificity. Additionally, the BOVIGAM test detects lower levels of bIFNγ compared to the ELISA proposed in this study. As noted by previous authors, tuberculosis diagnosis is influenced by several factors, including: (1) inadequate sample quantity and volume, (2) utilization of the same sample for multiple diagnostic tests, which may lead to poor microorganism distribution, (3) sample location, and (4) inefficiencies in sample processing techniques worldwide (Carrizosa-Urbina, 2015 & 2019). The Kappa coefficient (κ) analysis yielded a value of 0.49, with a 95% confidence interval (0.39-0.58), indicating a moderate level of agreement between the two diagnostic tests. Statistical analysis was performed using a non-parametric test for paired nominal variables with a binomial distribution, assessed through McNemar's Chi-square test ($\chi^2=6$, $p=0.0143$, $p<0.05$). This result provides evidence of a statistically significant difference in the identification of positive cases between the two methods. According to Gilchrist, as cited by Carrizosa-Urbina (2015), a Kappa value between 0.40 and 0.75 suggests moderate agreement; therefore, the obtained value of 0.49 supports an acceptable level of concordance between the tests (Gilchrist, 2009; Carrizosa-Urbina, 2015).

The evaluation of the likelihood ratio (LR) revealed an LR+ of 13.87 and an LR− of 0.33, placing the tests within the established parameters (LR+ >10 and LR− <0.5). This indicates that the capture ELISA test for bIFNγ is a useful diagnostic tool, as an individual infected with *Mycobacterium bovis* is 13 times more likely to test positive through the capture ELISA than a non-infected individual (Figure 4, Fagan Nomogram for the Likelihood Ratio of the Evaluated Tests).

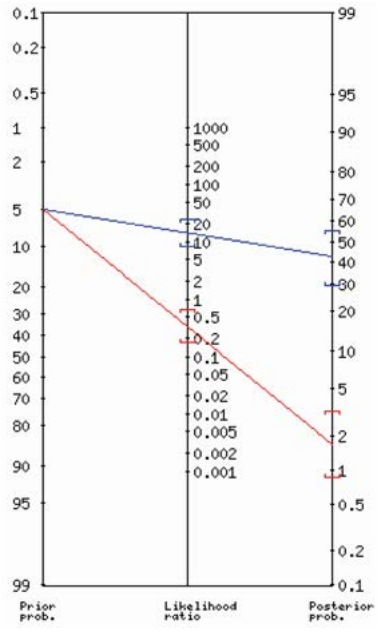

Figure 4. Fagan Nomogram for the Likelihood Ratio of the Evaluated Tests. This analysis aims to determine the post-test probability of positive results in both the intradermal test and the capture ELISA, thereby validating the plausibility of the obtained results within the evaluated cattle population (n=384).

CONCLUSIONS

This study represents the first strategic approach to implementing a capture ELISA test for determining bIFNγ levels in bovine serum samples. The use of specific polyclonal antibodies targeting bIFNγ rather than commercial monoclonal antibodies was successfully validated within the capture ELISA assay. This approach leveraged recombinant antigen technology and hyperimmune sera as a diagnostic alternative for bovine tuberculosis. Biological samples were obtained from beef cattle, dairy cattle, and dual-purpose herds originating from at least three different geographic regions of Mexico. Once validated across multiple samples, the bIFNγ capture ELISA assay could be effectively applied to the control and eradication of bovine tuberculosis in Mexico. This study demonstrated that the concordance between the intradermal test and the capture ELISA immunoassay for diagnosing bovine tuberculosis at the national level is satisfactory. Furthermore, our results align with the current prevalence rates reported by national zoosanitary authorities regarding infection in Mexican herds. The development of domestically produced diagnostic tests could significantly reduce import costs and improve accessibility.

DATA AVAILABILITY

The extensive data presented in this study are available upon request from the corresponding author.

FINANCING STATEMENT

This study was conducted with support from the FESC-PAPIIT TA200221 program. Dr. VRA received research chair support from COMECyT-CAT2022-0125.

All authors have read and approved the published version of the manuscript.

ACKNOWLEDGMENTS

This project was supported by the FESC-PAPIIT TA200221 program. Dr. VRA received a research chair grant from COMECyT-CAT2022-0125. Additionally, support was provided by private ranches in the State of Mexico and collection centers, CEA UNAM FESC.

CONFLICT OF INTERESTS

None of the authors declare any conflict of interest regarding this publication.

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