Study on a supplemental test to improve the detection of bovine tuberculosis in individual animals and herds

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Abstract

Background

With a worldwide occurrence, bovine tuberculosis (bTB) is a zoonotic disease that is difficult to control, mainly due to the lack of a diagnostic testing to detect infected animals at all stages. Furthermore, the current standard diagnostic test, the Tuberculin Skin Test (TST), is logistically difficult and time consuming. To address this challenge, the aim of this study was to evaluate the sensitivity and specificity of a multiplex enzyme-linked immunosorbent assay (ELISA) comparing with the TST used for the diagnosis of tuberculosis in cattle in Brazil. The study included 400 Nelore females raised for beef on five farms, in different municipalities in Brazil. The comparative cervical test (CCT) was done and on the day of inoculation of the Purified Protein Derivative (PPD) blood samples were obtained and stored for further analysis of the ELISA IDEXX™ Mycobacterium bovis immunoassay.

Results

Lack of agreement between CCT and ELISA IDEXX™ was observed. No diagnosis described as positive reactor on the CCT was positive at the ELISA, indicating two false positive reactors and 22 negative reactors by CCT were positive by the ELISA IDEXX™. The ELISA IDEXX™ showed sensitivity significantly higher than the official CCT and no significant differences in specificity was observed. ELISA also detected infected animals and herds undetected by the CCT. The parallel use of CCT and ELISA increased sensitivity and the feasibility bTB screening, thus improving the cleaning of the herds.

Conclusions

The results obtained here suggest that the ELISA IDEXX™ may be a supplemental test for the detection of Mycobacterium bovis infection in regions without routine testing and slaughter, where the disease generally progresses to more advanced stages and antibody responses are likely to be more prevalent. The results provided evidence to support the validation of the ELISA IDEXX™ as a supplemental test for bTB eradication programs.

Background

Bovine tuberculosis (bTB), a zoonosis that affects humans, among other species (1, 2, 3, 4), is difficult to control due to the lack of effective vaccine, the presence of wildlife reservoirs, and the lack of a diagnostic assay with sufficient sensitivity (Se) and specificity (Sp) to detect sick animals at all stages of infection (3, 5).

Therefore, the success of bTB eradication and control programs is based on early detection and removal of reactors from a herd (6). Routine testing and cull strategy have been applied globally (3). Therefore,

screening-test accuracy is critical to eradication programs. The single intradermal cervical tuberculin (SCT) test or single intra-dermal comparative cervical tuberculin (CCT) test in Europe, the caudal fold tuberculin (CFT) test in North America, Australia and New Zealand (7), and the SCT and CFT tests in Brazil (6), are the prescribed test for international trade (8). From a practical point of view, the diagnostic performance, the feasibility of execution and practicality of each test, as well as the costs and associated biological risks, should be considered for better strategic use (7).

Since late 19th century, the Tuberculin Skin Test (TST) has been the primary antemortem test available to support bTB eradication campaigns (9, 10). Advantages of the TST and reasons for its wide use are low costs, high availability, long history of use and, for a long time, the lack of alternative methods to detect bTB (9, 10, 11, 12). On the other hand, this test has many known limitations, including difficulties in performance and result interpretation, need for a second-step visit, low degree of standardization, and reduced test accuracy (13).

Due to the TST limitations in terms of sensitivity and specificity, the credibility of the diagnosis is frequently questioned given the occurrence of false-positive and false-negative reactions, therefore, it is necessary to confirm reactive animals using other methods, ensuring the reliability of the diagnosis (5, 10, 6, 7). Research continues into the development of new, more accurate, more sensitive tests, less subject to the individual operative performance and subjective interpretation (14, 15, 16, 17, 18, 19). As a complementary technique to the intradermal test in the detection of antibodies to *Mycobacterium bovis* (*M. bovis*) in animals exposed to the agent, a serological test with good sensitivity and specificity would be a viable alternative (9).

Over the last few years, the potential for use of an antibody assays to detect *M. bovis* infection in cattle is being consolidated (16, 17, 18, 19, 20, 21, 22, 23, 24, 25). The ELISA have proven to be useful as ancillary serial (to enhance Sp) and parallel (to enhance Se) tests in several species (26, 27, 28, 29, 30). Moreover, a booster effect on the antibody response caused after injection of tuberculin has been reported and recommended as a strategic option to increase the sensitivity of serological assays (26, 27, 28). The ELISA using MPB83 and MPB70 antigens (IDEXX *M. bovis* Ab Test, IDEXX Laboratories, Westbrook, Maine, US) is an immune enzymatic assay promising sensitivity and specificities superior to most tuberculosis diagnoses for both primary and supplementary diagnosis in cases of inconclusive results of the diagnosis by simple or comparative cervical test (16, 31, 12).

The Brazilian guidelines for control and eradication of animal tuberculosis determines intradermal tuberculin testing as the standard method of diagnosing bTB (32). The primary screening is performed using the CFT test (beef only), and the SCT or the CCT test (dairy and beef). The CCT is also adopted as confirmatory test. Until 2019 no others ancillary indirect methods for bTB diagnosis were approved by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA/BRAZIL). Adoption of complementary field bTB diagnostic tests can improve the detection of infected animals and herds.

To the best of our knowledge, there is no *in vivo* study comparing CCT test with ELISA IDEXX™ to evaluate the last as supplemental test for bTB in beef cattle in Brazil. The objective of this study was to determine

the sensitivity (Se) and specificity (Sp) of the CCT test and a commercial ELISA multiple test (ELISA IDEXX™), as ancillary tests in beef cattle, under field conditions, using a Bayesian approach in order to provide evidence-based suggestions to the improvement of national bTB eradication programs.

Results

Prevalence of Tuberculin Skin Test x ELISA for the diagnosis of bovine tuberculosis in beef cattle

The SCT test (considering only the PPDb values), revealed positive animals in all the studied farms, with higher values on farm 4 followed by farms 3, 5 and 2, and with lower values on farm 1 (Table 1).

Table 1
SCT (PPDb), CCT and ELISA bTB results in 400 Nelore cattle by farm.

Farm	Exam % (n/n)					
	SCT (PPDb)*		CCT**		ELISA***	
	-	+	-	+	-	+
1	96.25 (77/80)	3.75^{c} (3/80)	100 (80/80)	0.0^{c} (0/80)	98.75 (79/80)	1.25^{c} (1/80)
2	92.50 (74/80)	7.50^{bc} (6/80)	100 (80/80)	0.0^{bc} (0/80)	93.75 (75/80)	6.25^{bc} (5/80)
3	86.25 (69/80)	13.75^{b} (11/80)	98.75 (79/80)	1.25^{b} (1/80)	90.0 (72/80)	10.0^{b} (8/80)
4	62.50 (50/80)	37.50^{a} (30/80)	98.75 (79/80)	1.25^{a} (1/80)	91.25 (73/80)	8.75^{a} (7/80)
5	88.75 (71/80)	11.25^{bc} (9/80)	100 (80/80)	0.0^{bc} (0/80)	98.75 (79/80)	1.25^{bc} (1/80)
TOTAL	**85.25 (341/400)**	**14.75^{A} (59/400)**	**99.50 (398/400)**	**0.50^{C} (2/400)**	**94.50 (373/400)**	**5.50^{B} (22/400)**

Different uppercase letters between lines and different lowercase lines between columns differed between them ($P < 0,0001$). *SCT (PPDb) – Simple Cervical Test, **CCT – Comparative Cervical Test, *** ELISA IDEXX™ *M. bovis*

The CCT test results showed that 2/400 animals were classified as positive (reactors). In the experiment carried out on farms 3 and 4, the CCT test results were similar. Out of 80 tested animals per farm, 1 animal in each of the herds had a positive result, however the CCT tested reactive animals were not

reactive on the ELISA test. On farms 1, 2 and 5, all animals were CCT tested non-reactive, therefore only 2/5 herds were considered positive for bTB according the CCT testing on this study. The results obtained by ELISA IDEXX™, however, were different from those indicated by the CCT tests. A total of 22 out of the 400 animals (5.5%) were considered positive. At the herd-level, all the 5 herds (100%) had at least one positive animal (Table 1).

As all negative animals with values ≤ 1.9 (32), it was possible to observe that the SCT tests had an occurrence of 14.75% (59/400) of positive animals, while the CCT test was 0.50% (2/400) and the ELISA detected 5.50% (22/400) ($P < 0.0001$). Note that the number of animals reacting to SCT test was greater than ELISA and CCT test. Similarly, there was a higher frequency of animals positive in the ELISA compared to the CCT test (Table 1).

When comparing the CCT and SCT tests, it was observed that of the 59 positive tests for SCT, only two were equally positive for CCT (Table 2). In the comparison of the CCT test with the ELISA, the two animals reacting to the CCT were negative to the ELISA, while 22 animals were positive to the ELISA, but negative to the CCT (Table 3). Regarding the prevalence of SCT x ELISA, it was possible to indicate that of the 59 animals positive for SCT, only six of them were equally reactive to ELISA. Another 16 animals were positive for ELISA, but not reactive to SCT (Table 4).

Table 2
Comparison of the results of the CCT x SCT (PPDb) in 400 Nelore cattle

		SCT (PPDb)		TOTAL
		Negative	Positive	
CCT	Negative	341 (85.25%)	57 (14.25%)	398 (99.50%)
	Positive	0 (0.0%)	2 (0.50%)	2 (0.50%)
TOTAL		341 (85.25%)	59 (14.75%)	400 (100.0%)
P < .0001; Kappa = 0.0564				

Table 3
Comparison of the results CCT x ELISA in 400 Nelore cattle

		ELISA		TOTAL
		Negative	Positive	
CCT	Negative	376 (94.0%)	22 (5.50%)	398 (99.50%)
	Positive	2 (0.50%)	0 (0.0%)	2 (0.50%)
TOTAL		378 (94.50%)	22 (5.50%)	400 (100.0%)
P < .0001; Kappa = -0.0093				

Table 4
Comparison of the results SCT x ELISA in 400 Nelore cattle

		ELISA		TOTAL
		Negative	Positive	
SCT (PPDb)	Negative	325 (81.25%)	16 (4.0%)	341 (85.25%)
	Positive	53 (13.25%)	6 (1.50%)	59 (14.75%)
TOTAL		378 (94.50%)	22 (5.50%)	400 (100.0%)
P < .0001; Kappa = 0.0739				

Sensitivity and Specificity of bTB tests (CCT x ELISA)

As in Brazil, the CCT is considered a confirmatory test, while the SCT is a screening test for beef cattle. It was decided to establish a comparative analysis of the sensitivity and specificity of the ELISA against the CCT.

Bayesian analysis showed that the Se of ELISA IDEXX™ was significantly higher (P = 0.003) than CCT's Se. On the other hand, no significant differences in Sp between CCT and ELISA IDEXX™ (Table 5).

The parallel interpretation of the results of the two tests, show an increase of Se at herd-level from 40% on the official CCT test to 100% and at animal-level from 0.5–6.00% (Tables 6 and 7).

Table 5
Apparent Prevalence, Sensitivity, and Specificity of bTB tests with 95% Confidence Interval

Parameter	Estimative	CI
AP	0.0033	(0.0005; 0.0087)
Se_{CCT}	0.7329	(0.5986; 0.8497)
Se_{ELISA}	0.8882	(0.8063; 0.9513)
$Se_{CCT} - Se_{ELISA}$	-0.1553	(-0.3049; -0.0158)
Sp_{CCT}	0.9557	(0.9372; 0.9718)
Sp_{ELISA}	0.9479	(0.9256; 0.9665)
$Sp_{CCT} - SP_{ELISA}$	0.0078	(-0.0186; 0.0357)
AP – Apparent Prevalence; Se_{CCT} – Comparative Cervical Test Sensitivity; Se_{ELISA} – ELISA IDEXX™ Sensitivity; Sp_{CCT} – Comparative Cervical Test Specificity; Sp_{ELISA} - ELISA IDEXX™ Specificity		

Table 6
Single and parallel interpretation of CCT + ELISA IDEXX™ at Herd-level in 400 Nelore cattle.

Results	Tests		
	CCT*	ELISA**	CCT + ELISA
Positive	**40%** (2/5)	**100%** (5/5)	**100% (5/5)**
Negative	**60%** (3/5)	**0%** (0/5)	**0% (0/5)**
*CCT – Comparative Cervical Test, ** ELISA IDEXX™ *M. bovis*			

Table 7
Single and parallel interpretation of CCT + ELISA IDEXX™ at Animal-level in 400 Nelore cattle.

Results	Tests		
	CCT*	ELISA**	CCT + ELISA
Positive	**0,50%** (2/400)	**5,50%** (22/400)	**6%** (24/400)
Negative	**99,50%** (398/400)	**94,50%** (378/400)	**94%** (376/400)
*CCT – Comparative Cervical Test, ** ELISA IDEXX™ *M. bovis*			

Discussion

This study assessed the performance of bTB test routinely used in eradication programs (SCT and CCT tests) and a potential supplemental test (ELISA IDEXX™) under field conditions in Brazil using a Bayesian approach.

ELISA IDEXX™ presented higher Se than CCT testing even in the absence of the booster effect. The evaluation of experimental diagnostic techniques for the detection of antibodies against *M. bovis* has demonstrated that depending on the epidemiological situation the Se of the antibody detection tests in the absence of the booster effect could be even bigger than the one obtained using official techniques that detect the cell-mediated immune (CMI) response (27, 28). Recently, under a high bTB prevalence situation, serological tests presented higher Se than official techniques in the absence of booster effect (17, 29). In our study, adopting a parallel interpretation the apparent prevalence ranged from 1.25% – 11.25% within the herds and may explain the higher Se presented by ELISA IDEXX™.

No overlap was found between the 22 animals with positive ELISA IDEXX™ and the two animals with positive CCT test results agreeing with similar findings of other studies (33, 34). On the other hand, in this study six animals' (1.50%) reactors on the SCT test were positive on ELISA IDEXX™, which differ from another study where no agreement was found between SCT testing and ELISA IDEXX™ (33). The lack of or low agreement between the positive results of the two tests may reflect different elements of the

immune response (humoral and cell-mediated immunity) (33, 34). The detection of CMI response to infection with *M. bovis*, as assessed by the TST usually fail to detect chronic stages of the infection (26, 29, 35).

Particularly in situations such as in the area where the research was carried out, with absence of a test-and-slaughter routine, disease will progress to more advanced stages and antibody responses are likely to be more prevalent (Table 1). Moreover, a factor that may have influenced our results might be the age. All tested animals were adult females older than 24 months, unfortunately no further information was obtained allowing us to determine the mean age of the animals by farm, which is a limitation of this study.

Under the study's circumstances no decisions regarding culling animals should be made based only on the SCT test, since the CCT confirmatory test would confirm as positive only 2/59 reactors at the first screening. On the other hand, aiming to avoid additional visits to the farm, veterinarians might adopt the strategy of using the CCT test solely. This strategy applied to our study would result in only 2 two herds and 2 two animals considered bTB positive. In its turn, considering only the ELISA, all herds would be classified as bTB positive and 22 CCT negative animals would be diagnosed as bTB positive. Therefore, the sole use of CCT testing, would leave behind three infected herds and 22 false negative animals increasing the risk of the disease spreading. The potential of the serological tests to identify non-reactive TST results is being reported and suggests that their application to test non-infected herds would help to increase the performance of the screening strategy in current bTB eradication programs (19, 25, 36).

The application of a parallel testing strategy SCT + ELISA on the numbers of this study would result in no increase on the proportion of infected herds (5/5), however would result in an increase in the proportion of a positive rate at animal level (75/400). Therefore, in regions with high bTB prevalence and no indemnity for cattle owners, such as from this study, seems to not be reasonable in the adoption of the above procedure since it notably would increase the false positive animals leading to unjustifiable economical loss and ultimately low adherence or even avoidance to eradication programs. On the other hand, the parallel strategy CCT + ELISA, would result in an increase on the proportion of herds infected by 60% and at animal level the increase of positive animals by 5.5%, these results are compatible with previous studies (17, 25, 36). Thus, the parallel interpretation of the diagnostic techniques that detect cellular and humoral immune response although can be recommended as supplemental test to detect false negative TST animals (17, 25, 33, 34, 36) should be considered according the local circumstances.

Although, the study design does not allow the assessment of ELISA as an ancillary serial test, the Bayesian analysis show no difference in Sp between CCT testing and ELISA IDEXX™ (Table 5). Thus, the diagnostic power of animals truly negative for bTB is confirmed by the CCT test, standing out as a good confirmatory test for the diagnosis of *M. bovis* infection. Additionally, a trial to test a serial diagnosis scheme would allow to confirm the occurrence of a booster effect in beef cattle.

A study to assess the reliability of the combination CFT test (the usual screening test for beef cattle), ELISA in parallel and serial schemes, and isolation of the *M. bovis* by culture (the golden standard) would

be of a great value. Adoption of supplemental tests would represent significant logistical improvements on the bTB program, reducing farm visits, CCT test reading errors, time for removing the infection from the herd, and allowing to store serum samples for confirmatory tests for a long period of time. Furthermore, the animals would have to be reunited and handled once, which would be an extra advantage when working with beef, representing a great benefit for the farmers and the veterinarians.

Although the study needs to be extended, circumstantial evidence was obtained to support the recommendation of adoption of serological tests as supplemental to the traditional TST schemes as an alternative strategy that may contribute to accelerated bTB eradication helping in outbreak management and disease control.

Conclusions

The ELISA IDEXX™ multiplex presented significant lower Se than the official TST and SCT test, and higher Se than the CCT test.

The ELISA IDEXX™ detected infected animals and herds missed by the CCT test. Parallel use of CCT test and ELISA increased the sensitivity, the feasibility of screenings for *M. bovis* infection diagnosis, and speed up the cleansing of herds.

The ELISA IDEXX™ can be a supplemental test for *M. bovis* infection detection in regions with no test-and-slaughter routine where the disease usually progress to more advanced stages and antibody responses are likely to be more prevalent.

Results provided evidence to support the validation of a serological test as supplemental to the official TST on the Brazilian eradication program.

Methods

Study Design

This was a cross-sectional study, designed to assess the feasibility of a supplemental test to improve the detection of bovine tuberculosis in individual animals and herds. A total of 400 female Nelore (*Bos taurus indicus*), aged over 24 months, raised on farms in Para State, Brazil, were included in the study. The animals were selected from a population of 7,600 cows, included in the study were cows older than 24 months of age, not pregnant, with no history of abortions and with a body condition score of (BSC) > 3. The cows came from five farms located in the following municipalities: Capitao Poco (Farm #1, n = 80 of 2,500 cows), Garrafao do Norte (Farm #2, n = 80 of 700 cows), and Sao Francisco do Para (Farm #3, n = 80 of 600 cows), in Para's northeastern region and Castanhal (Farm #4, n = 80 of 1,300 cows), and Santa Izabel (Farm #5, n = 80 of 2,500 cows) from Belem's metropolitan mesoregion. The sampling was carried out from March 13 to May 4, 2013.

Blood collection

Samples were collected from all cows in the experimental group composed of cows from each farm. There was 10.0 mL of blood collected by puncture of the external jugular vein, without excessive tourniquet of the vessel, using siliconized vacutainer tubes without anticoagulant and properly identified. The samples were centrifuged for 15 minutes at a speed of 3,000 G, then separated by aspiration of the serum, aliquoted in 2 mL Eppendorf microtubes, identified and stored at -20 ºC for subsequent serological testing (ELISA).

Testing

Enzyme-Linked Immunosorbent Assay – ELISA

The ELISA IDEXX™ *M. bovis* was performed as described previously (16) and according to the manufacturer's instructions. Shortly, two microtiter plate wells were used for the positive control, two for the negative control, and a blank well to reference the microplate reader. The reading was performed on a TP Reader Basic/Thermoplate microplate reader, at a wavelength of 450 nm at an accuracy of ± 2 nm and an absorbance resolution of 0.001A at an accuracy of ± 0.03A. The results of optical density (OD) provided by the reader were recorded and used to calculate the validation of the test and then the results of the samples according to the specifications of the kit manufacturer.

Comparative Cervical Tuberculin (CCT) Skin Test

On the same day of blood collection, inoculations of avian (PPDa) and bovine (PPDb) tuberculin were performed intradermally, at a dose of 0.1 mL in the cervical region, in places previously demarcated by hair removal, at 15 to 20 cm between the two inoculations. PPDa was inoculated cranially and PPDb caudally, on the same side of all animals in the herd to be tested (8). The skin thickness of the inoculation site was measured using calipers before injection. Test results were determined by the same researcher at 72 h post-injection by measuring the increase in skinfold thickness. Data for analysis of the SCT were obtained from reading only the inoculated PPDb. Interpretations of the test results were made according to the Brazilian standard for screening tests for bovine tuberculosis (32).

Statistical analysis

The descriptive statistics of the data, represented by the frequencies (%) of reagents or non-reagents animals, both between the referred exams (ELISA, CCT. and SCT), between the different farms (1, 2, 3, 4 and 5) and interaction Exam*Farm, was obtained by Freq procedure of the SAS program (SAS® 9.3, SAS Institute Inc., Cary, NC, USA).

Inferential statistical analyses were performed using analysis of variance (ANOVA), with the Glimmix procedure, from the SAS program. Considering that the three exams (ELISA and CCT and SCT) were performed on the same animal, the statistical model was constituted by the Exam and Farm classificatory variables.

Agreement between diagnostic outcomes (positive vs. negative) from 2 by 2 exams (CCT vs. SCT (PPDb); CCT vs. ELISA; and SCT (PPDb) vs. ELISA) were evaluated by the Kappa coefficient with Freq procedure

of SAS. Interobserver agreement was also evaluated by the weighted kappa coefficient with Freq procedure of SAS. The significance level of 5% was used for both LSMeans and Kappa analyses.

A Bayesian method was used to estimate the Se and Sp of the CCT test and ELISA in the absence of a gold standard. We followed the conservative assumption that results were not independent. Additionally, the single and parallel interpretations of CCT + ELISA IDEXX™ were done to show the Animal and Herd-level of sensitivity.

The comparison between the frequencies of the groups was performed using the Least Square Means test (LSMeans) of the SAS. The significance level of 5% was used.

Abbreviations

bTB – Bovine Tuberculosis CCT – Comparative Cervical Tuberculin CFT – Caudal Fold Tuberculin ELISA – Enzyme Linked Immunosorbent Assay ELISA IDEXXTM – Enzyme Linked Immunosorbent IDEXX Laboratories MAPA/BRAZIL – Brazilian Ministry of Agriculture, Livestock and Food Supply PPD – Purified Protein Derivative PPDa – Purified Protein Derivative avian PPDb – Purified Protein Derivative bovine SCT – Single Intradermal Cervical Tuberculin Se – Sensitivity Sp – Specificity TST – Tuberculin Skin Test

Declarations

Ethics approval and consent to participate

The study was submitted for and approved by the Ethics Committee on the Use of Animals of the Amazon Federal Rural University (UFRA), Belem, Brazil

Consent for publication-was this obtained?

The informed consent for publication by the ranchers, was obtained prior to the experiment. For ethical reasons, the authors chose not to identify nominally the farms included in this study.

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

JBK, PAMC, RBV, BMM, JDRF, RSJ and DCS contributed to the study design and drafted the manuscript; EMS, DCS, ASC and ASLK were responsible for sampling, laboratory testing, and tabulated the data; BMM and MRMT conducted the statistical analyses and drafted the manuscript. All authors read and approved the final manuscript

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