



Diagnostic performance of a commercial ELISA used as a complementary test for bovine tuberculosis in two bovine herds with different disease status

[Desempenho diagnóstico de um ELISA comercial usado como teste complementar para tuberculose bovina em dois rebanhos bovinos com diferentes estágios de controle da doença]

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ABSTRACT

Bovine tuberculosis is a worldwide spread zoonotic disease. Intradermal tuberculinizations are the most used diagnostic tests in the world. Serological tests can be an ancillary diagnosis for bovine tuberculosis. The objective of this study was to evaluate the diagnostic performance of the ELISA *Mycobacterium Bovis* Antibody Test Kit IDEXX™ in infected herds, which were in different disease control stages. One hundred and twenty animals from two dairy herds of Minas Gerais state, Brazil, were subjected to the ELISA serological test and the comparative cervical tuberculin test (CCT). Diagnostic test parameters were estimated using Bayesian latent class models and concordance between tests estimated by the frequentist approach. The ELISA test presented lower sensitivity than CCT in both herds. Its sensitivity was higher in the herd in sanitation process. Specificity estimates were above 95% in both herds. *Kappa* index indicated low concordance or even disagreement between tests. According to the results, the ELISA IDEXX should not be used as substitution for CCT. The tests must not be associated in series. Parallel association increased diagnostic sensitivity in the herd which was in the process of sanitation.

Keywords: comparative cervical tuberculin test, tuberculosis serology, diagnostic test evaluation

RESUMO

A tuberculose bovina é uma zoonose de distribuição mundial cujos testes mais utilizados para o diagnóstico são as tuberculinizações intradérmicas, simples e compartivas. Contudo, testes sorológicos podem constituir diagnósticos auxiliares. O objetivo deste estudo foi avaliar o desempenho diagnóstico do teste ELISA *Mycobacterium Bovis* Antibody Test Kit IDEXX® em rebanhos bovinos infectados, que se encontravam em diferentes estágios de controle da doença. Cento e vinte animais de dois rebanhos leiteiros provenientes do estado de Minas Gerais-Brasil foram submetidos ao ELISA e à tuberculinização cervical compartiva (TCC). Avaliou-se o desempenho dos testes por meio de modelos Bayesianos de classe latente e a concordância entre os eles, por meio de estatística frequentista. Uma maior sensibilidade do teste foi observada no rebanho previamente tuberculinizado. Em ambos os rebanhos o TCC foi mais sensível que o ELISA. Especificidade acima de 95% foi encontrada em ambos os rebanhos. Foram observadas baixa concordância ou mesmo discordância entre os testes. De acordo com os resultados obtidos, o teste ELISA-IDEXX não deve ser utilizado em substituição à TCC, tampouco devem ser associados em série. Houve aumento da sensibilidade quando os testes foram associados em paralelo no rebanho que já se encontrava em processo de saneamento.

Palavras-chave: tuberculinização cervical comparativa, sorologia para tuberculose, avaliação de testes diagnósticos

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INTRODUCTION

Bovine tuberculosis is a globally distributed zoonosis that poses a considerable risk to public health and causes significant economic losses in livestock production. It is a chronic, asymptomatic disease caused mainly by *Mycobacterium bovis*. Its control is based on the identification and the timely elimination of infected animals (Bovine..., 2017). Intradermal tuberculinizations are the most used *in vivo* diagnostic tests for bovine tuberculosis in the world. These tests are based on the cellular immune response to tuberculous proteins present in allergens (Casal *et al.*, 2014).

Despite being widely accepted, there are intrinsic characteristics which may lead to false-positive and false-negative reactions in these tests (Bezoz *et al.*, 2014). Thus, complementary diagnostic tests are necessary to correctly identify the largest possible number of infected animals, leading to a faster, cheaper and safer sanitation process (Casal *et al.*, 2014). Tuberculosis serological tests do not present the logistic drawbacks of the tests based on the cellular immunity and is simpler to perform. They can identify animals at different stages of the disease, depending mainly on the antigens used in the test. The *Mycobacterium Bovis* Antibody Test Kit - IDEXX™, USA, is a recommended diagnostic test for bovine tuberculosis which uses MPB70 and MPB83 proteins as immobilized antigens in the solid (Waters *et al.*, 2011; Bovine..., 2017).

Some traditional textbooks on diagnostic testing still refer to the test sensitivity and specificity as values that are intrinsic to the diagnostic test. Nevertheless, both diagnostic test parameters may vary with external factors (Berkvens *et al.*, 2006). The present work aimed to evaluate the diagnostic performances of the *Mycobacterium Bovis* Antibody Test Kit IDEXX™ in two tuberculous dairy herds with different disease status using Bayesian latent class analysis (BLCA) and frequentist approaches.

MATERIAL AND METHODS

One hundred and twenty animals from two dairy herds were tested. Tuberculosis had never been suspected in both herds until the sale of animals, later identified as reactive in comparative cervical tuberculin (CCT), performed in the

destination herds. After that, procedures to control the disease began in one of the herds. This herd, named herd 1, is in the municipality of Machado, in the southern region of the state of Minas Gerais, Brazil. Herd 1 was composed of 79 Holstein animals, which had already been in the process of sanitation for more than a year before the time of blood collection for the ELISA test. At least two CCT tests, 60 to 90 days apart, have been done with removal of positive animals. Some of these animals showed lesions suggestive of tuberculosis at slaughtering.

The other herd, named herd 2, is in the municipality of Leopoldina, in the Zona da Mata region of the same state, and consisted of 41 crossbred Holstein-zebu (Gir) animals. This herd had not started the sanitation process until we had visited it to collect blood and follow tuberculinization. Comparative cervical tuberculinization (CCT) was carried out by private veterinarians licensed by the official animal health service in both herds, according to the Brazilian national program for the control and eradication of brucellosis and tuberculosis (Programa Nacional de Controle e Erradicação de Brucelose e Tuberculose (PNCEBT), Ministério da Agricultura, Pecuária e Abastecimento - MAPA).

Inoculation sites for avian PPD (PPDa) and bovine PPD (PPDb) were prepared by trichotomy of an area in the middle third of the neck or in the region of the scapula. Skin thickness at injection site was measured before PPD injections and at 72±6h post-inoculation by the same veterinarian using the same calipers. The animals were inoculated with 0.1ml of each PPD, 2000UI/mL at least, at the center of the inoculation sites. The results were interpreted according to Table 1 (Brasil, 2017). CCT inconclusive animals were included in the models considering them either as positive and negative results in statistical analysis (Shinkins and Thompson, 2013).

Whole blood from all animals was collected at the time of the last tuberculinization in herd 1. Time interval from the previous tuberculinization until blood collection was 60 - 90 days. Blood collection occurred at the time of the first tuberculinization in herd 2. Serum, obtained after blood clotting, was aliquoted, uniquely identified and stored at -20°C until the tests were performed.

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Table 1. Comparative cervical tuberculin test (CCT) interpretation criteria for bovine tuberculosis diagnosis, according to the Brazilian National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT)

	$\Delta B - \Delta A$ (mm)	Result Interpretation
$\Delta B < 2,0\text{mm}$	-	Negative
$\Delta B < \Delta A$	< 0	Negative
$\Delta B \geq \Delta A$	0.0 a 1.9	Negative
$\Delta B \geq \Delta A$	2.0 a 3.9	Inconclusive
$\Delta B \geq \Delta A$	≥ 4.0	Positive

ΔB = skin fold thickness increase 72 hours after bovine tuberculin inoculation, ΔA = skin fold thickness increase 72 hours after avian tuberculin inoculation—source: Brasil, 2017.

Serum samples from all animals were subjected to the *Mycobacterium Bovis* Antibody Test Kit IDEXX™, USA. The tests were performed according to the manufacturer's instructions. Results were expressed as the ratio of the sample optical density (OD) minus the mean kit negative control OD to the mean kit positive control OD minus the mean kit negative control OD (S/P ratio). A positive result was defined by the manufacturer as an S/P ratio ≥ 0.3 , and a negative result as an S/P ratio < 0.3 .

Diagnostic test's sensitivities and specificities were estimated using Bayesian latent-class models. Initially, a two tests-two herds (populations) model was proposed. After the results obtained with the first model, models for two tests-one herd were used. Models were run in WinBUGS program, version 1.4 (Lunn *et al.*, 2009). Two chains were run in each model, with 100,000 iterations in each one. The first 5,000 iterations were discarded as burn-in. The convergence of the Markov chains was checked by visual inspection of their history and by verification of the Monte Carlo error (Hamra *et al.*, 2013). One in 10 generated values was taken for analysis in order to minimize auto-correlation.

Model Beta (α , β) was used as *a priori* distribution for the sensitivity, specificity and prevalence parameters (Joseph *et al.*, 1995). Priors were obtained from similar literature to this study or from the specialists' opinions (Joseph *et al.*, 1995). The criterion for selecting specific papers was the similarities with this study in terms of diagnostic tests' methodologies, objectives, type of samples, and preferably, those carried out in Brazil. In the case

of not meeting this specific condition, international literature was used.

Expert opinions and information obtained from the anamnesis of the herds, tuberculinization follow-up and the occurrence of tuberculosis suggestive lesions found that at the time of the slaughtering some animals were considered to define the values of the hyper-parameters' *a priori* distributions for prevalence (Table 2). Tests were considered conditionally independent because ELISA and tuberculinization are based on different biological principles (Gardner *et al.*, 2000).

Agreement and discordance between tests were evaluated by the *kappa* test and McNemar chi-square or binomial test, according to pre-established criteria for the application of each test. The performance parameters of the tests associated in series and in parallel were also calculated (Siegel, 1975; Noordhuizen *et al.*, 2001). Animal use was approved under protocol 77/2016 - Committee of Ethics in the Use of Animals (CEUA) of the Federal University of Minas Gerais (UFMG).

RESULTS

The frequency of ELISA and CCT results are shown in Table 3.

The specificities and the sensitivities for each test in each herd, estimated through two tests-one herd models, statistics for concordance between tests and tuberculosis prevalence in each herd are presented in Table 4 and 5.

Results of the association of the tests in parallel and in series are presented in Table 6 and 7.

Table 2. *A priori* information for the validation parameters of comparative cervical tuberculin test (CCT) and ELISA for bovine tuberculosis diagnosis, and for the prevalence of bovine tuberculosis in herd 1 and herd 2

Herd	Test	Parameter	<i>a priori</i> information (95% lower-upper limits)	Source of information
1		Prevalence	5.0% (0.5% - 10.0%)	Paulo M. S. Filho / Alberto Knust Ramalho (LANAGRO-MG)
2		Prevalence	45.0% (30.0% - 60.0%)	Paulo M. S. Filho (LANAGRO-MG) Antônio Cândido Cerqueira Leite Ribeiro (EMBRAPA)
1, 2	CCT	Sensitivity	79.4% (60.9% - 93.8%)	(Lopes <i>et al.</i> , 2012)
		Specificity	97.7% (95.8% - 98.9%)	(Lopes <i>et al.</i> , 2012)
	ELISA	Sensitivity	63.0% (30.0% - 91.0%)	(Waters <i>et al.</i> , 2011)
		Specificity	95.0% (93.23% - 100.0%)	(Waters <i>et al.</i> , 2011)

*Mycobacterium Bovis Antibody Test Kit IDEXX™

Deviance Information Criterion (DIC) was used to evaluate the quality of data fit to the models (Spiegelhalter *et al.*, 2002).

Table 3. Cross tabulation of results of comparative cervical tuberculin test (CCT) and ELISA IDEXX* for the detection of antibodies against *Mycobacterium bovis* in two bovine herds with different disease status, considering the inconclusive animals in the CCT as negative and positive in turn

CCT Inconclusive = Negative				CCT Inconclusive = Positive			
Herd 1**	ELISA	+	-	Herd 1	ELISA	+	-
CCT	+	07	71	CCT	+	05	71
		07	71			05	71
Herd 2***	ELISA	+	-	Herd 2	ELISA	+	-
CCT	+	04	21	CCT	+	02	26
		04	21			02	11

*Mycobacterium Bovis Antibody Test Kit IDEXX™; **herd in tuberculosis sanitation process with previous tuberculinizations; *** herd not tuberculinized previously.

A posteriori estimates of the ELISA sensitivity was biased towards herd 2 when the model for two tests and two herds was used. Such behavior was not observed in relation to the specificity of the tests.

Table 4. *A posteriori* values of the sensitivity and specificity, kappa index and binomial test for the comparative cervical tuberculin test (CCT) and ELISA IDEXX* for the bovine tuberculosis diagnosis in herd 1, considering the inconclusive animals in the CCT as negative and positive in turn

	CCT Inconclusive = Negative		CCT Inconclusive = Positive	
	ELISA	CCT	ELISA	CCT
Sensitivity	60.22% (28.63–86.71) ^a	75.49% (55.87–89.88) ^a	61.79% (31.88–86.95) ^a	76.40% (57.65–90.35) ^a
Specificity	94.78% (90.79–97,54) ^a	97.69% (96.14 - 98.77) ^a	95.77% (92.15– 98.21) ^a	97.55% (95.93–98.70) ^a
Kappa Index	-2.3% (-6.2–1.7) ^b		18.8% (-18.8–55.0) ^b	
Binomial Test	P= 0.0820 ^{ns}		P= 0.453 ^{ns}	
Prevalence	3.32% (1.10–7.33) ^a		4.17% (1.56–8.54) ^a	
DIC	12.247		10.46	

* Mycobacterium Bovis Antibody Test Kit IDEXX™; ^a 95% credible interval (Bayesian statistics); ^b 95% confidence interval (frequentist statistics); DIC - Deviance Information Criterion, ^{ns}-non significant (5% significance bicaudal).

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Table 5. *A posteriori* values of the diagnostic test performance parameters, *Kappa* index and McNemar Qui-squared test for comparative cervical tuberculin test (CCT) and ELISA IDEXX* for the bovine tuberculosis diagnosis in herd 2, considering the inconclusive animals in the CCT as negative and positive in turn

	CCT Inconclusive = Negative		CCT Inconclusive = Positive	
	ELISA	TCC	ELISA	TCC
Sensitivity	25.87% (11.26–44.94) ^a	76.30% (59.97–89,25) ^a	21.63% (10.45–36.52) ^a	85.16% (73.12–93.89) ^a
Specificity	96.02% (91.35–98.78) ^a	97.36% (95.53–98.63) ^a	96.32% (95.47–98.61) ^a	97.33% (91.78–98.93) ^a
<i>Kappa</i>	-18.5% (-34.5% - 0.25) ^b		-5.5% (-20.4–9.4) ^b	
X ² McNemar	6.05 (p=0.0139)		18,89 (p=0.0001)	
Prevalence	47.45% (35.35–60.15) ^a		58.97% (47.78–69.95) ^a	
DIC	20.103		21.367	

* Mycobacterium Bovis Antibody Test Kit IDEXX™; ^a credible interval 95% (Bayesian statistics); ^b confidence interval 95% (frequentist statistics); DIC - Deviance Information Criterion.

Table 6. Sensitivity and specificity estimates of the CCT and IDEXX ELISA* parallel association for the diagnosis of bovine tuberculosis in two cattle herds with different control status of the disease

Herd	Parameter	CCT Inconclusive = Negative	CCT Inconclusive = Positive
1**	Sensitivity	90.25%	90.98%
	Specificity	92.59%	93.42%
2***	Sensitivity	82.43%	88.34%
	Specificity	93.48%	93.74%

* Mycobacterium Bovis Antibody Test Kit IDEXX™; **herd in tuberculosis sanitation process with previous tuberculinizations; *** herd not tuberculinized previously; CCT–Comparative Cervical Tuberculin Test; Parallel Sensitivity = 1 - (1- CCT Sensitivity)*(1- ELISA Sensitivity); Parallel Specificity = CCT Specificity * ELISA Specificity

Table 7. Sensitivity and specificity estimates of the CCT and IDEXX ELISA* associated in series for the diagnosis of bovine tuberculosis in two cattle herds with different control status of the disease

Herd	Parameter	CCT Inconclusive = Negative	CCT Inconclusive = Positive
1**	Sensitivity	45.46%	47.20%
	Specificity	99.88%	99.89%
2***	Sensitivity	19.77%	18.42%
	Specificity	99.89%	99.90%

* Mycobacterium Bovis Antibody Test Kit IDEXX™; **herd in tuberculosis sanitation process with previous tuberculinizations; *** herd not tuberculinized previously CCT–Compative Cervical Tuberculin Test; Serial sensitivity = CTC sensitivity*ELISA sensitivity; Serial Specificity = 1 - (1-CCT specificity) * 1 - (1-ELISA specificity).

DISCUSSION

Bayesian latent class models had already been used before to evaluate diagnostic tests for bovine tuberculosis (Álvarez *et al.*, 2012; Lopes *et al.*, 2012). However, it had never been done before for this commercial serological test, aimed to diagnose bovine tuberculosis in Brazil using BLCA. BLCA was an appropriate approach for this study due to the lack of a gold standard for the bovine tuberculosis diagnosis

and the uncertainties about herds' true prevalence. Hence, BLCA incorporated those uncertainties in the analysis (Joseph *et al.*, 1995; Alvarez *et al.*, 2012).

When using a two tests-two populations model, the posterior probability distribution of the ELISA's sensitivity was biased towards the herd of higher prevalence (herd 2), which indicates that the sensitivity of this diagnostic test could vary depending on the population's prevalence

(Toft *et al.*, 2005). This dependence may be related to the different stages of the disease in animals of the two herds.

Although the two tests-one population models were initially non-identifiable because they presented a greater number of parameters to be estimated than the number of degrees of freedom, the use of informative *priors* contributed to re-establish their identifiability conditions (Joseph *et al.*, 1995). The lower values of DIC found for these models also indicated that they were more adequate to the data than the model of two tests and two populations (Spiegelhalter *et al.*, 2002). Both tests were very specific, with small variations between herds and within herds. Also, specificities estimates were close to those already reported in the literature (Lopes *et al.*, 2012; Casal *et al.*, 2014).

The low sensitivity values for CCT found in this study are in agreement with those reported in the literature too (Bezoz *et al.*, 2014). Nevertheless, lowering the CCT cut-off to consider inconclusive animals as positives improved its sensitivity in herd 2, without any reduction in specificity. The same did not happen in herd 1. Albeit ELISA IDEXX was less sensitive than CCT, the test was able to identify reactive animals, possibly anergic ones or those in the early stages of the disease, which were not detected by CCT (Lightbody *et al.*, 1998). This ability is conferred by the adsorbed antigens MPB 83, a constitutive protein of *M. bovis* that induces early antibody response in infected animals, and MPB 70, a protein secreted by the bacterium at a later stage of the disease which induces late antibody response (Waters *et al.*, 2006; 2011). The very low or negative values found for the *Kappa* statistics indicates a low agreement or even a disagreement between the tests and corroborates the capacity of these two tests to identify animals in different stages of the disease.

According to the results obtained, the use of ELISA and CCT in parallel was a viable alternative to increase the sensitivity of tuberculosis diagnosis in herd 1, as was found by other authors (Casal *et al.*, 2014). This measure would accelerate the process of sanitation and

eradication of the disease, as it reduces the possibility of infected animals not detected by CCT to remain in the herd (Casal *et al.*, 2014). On the other hand it was not a good diagnostic strategy for herd 2.

The very low sensitivity of the tests when associated in series discourages this combination, although the resulting specificity rises close to 100%. It is known that the tuberculinization of infected animals induces humoral anamnestic immune response, mediated predominantly by IgG1. In non-tuberculinized infected animals, IgG2 predominates (Lightbody *et al.*, 1998; Waters *et al.*, 2011). This could be another factor contributing to the lower sensitivity of the ELISA in herd 2, since this herd had not started the sanitation process and could have a large number of infected animals with low levels of specific IgG1 circulating antibodies and high levels of IgG2, undetected by the serological test.

According to Lightbody *et al.* (1998) and Casal *et al.* (2014) the blood collection 15 days after tuberculinization substantially improves the sensitivity of the ELISA IDEXX™. However, this could not be done in this work because it would demand one more visit to the farm, in addition to the two visits to perform the CCT, would change the management of the herd, raise the costs of the diagnosis, which would certainly be refuted by the herd's owners. Therefore, blood was sampled on the day of tuberculin inoculation or on the day of registering the results of the CCT to overcome those drawbacks.

The sensitivity estimates of the ELISA found for herd 1 were similar to those already reported by other authors when blood was collected 15 days after tuberculinization. In herd 2, on the other hand, the lower values were close to those reported when blood for ELISA was collected on the day of tuberculinization (Casal *et al.*, 2014). Disparities in the sensitivity of the ELISA *Mycobacterium Bovis* Antibody Test Kit IDEXX® - EUA due to geographic differences were also reported (Troost *et al.*, 2016; Waters *et al.*, 2011). However, this does not appear to be the cause of the differences in diagnostic performances observed in this study because herds 1 and 2 come from very close regions within the same state in Brazil.

CONCLUSIONS

The use of the ELISA IDEXX (*Mycobacterium Bovis* Antibody Test Kit IDEXX® - EUA) for detecting antibodies to *M. bovis* MPB83 and MPB70 proteins had better diagnostic performance in bovine herd 1, which had already been in the process of sanitation, and was subjected to cervical tuberculinizations previously to serological tests. The serological test used at the beginning of the sanitation process, at the time of the first tuberculinization, did not improve the tuberculosis diagnosis in herd 2, due its very low sensitivity in this situation. According to the results obtained in this study, the ELISA IDEXX cannot be used in substitution for CCT, nor can the tests be associated in series. Nevertheless, parallel association improved tuberculosis diagnosis in herd 1. Considering inconclusive animals in CCT as positives was more effective to improve diagnostic sensitivity in herd 2 than the use of ELISA IDEXX in parallel association to CCT.

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