

Parameter estimation and use of gamma interferon assay for the diagnosis of bovine tuberculosis in Brazil¹

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ABSTRACT. Lopes L.B., Alves T.M., Stynen A.P.R., Mota P.M.P.C., Leite R.C. & Lage A.P. 2012. **Parameter estimation and use of gamma interferon assay for the diagnosis of bovine tuberculosis in Brazil.** *Pesquisa Veterinária Brasileira* 32(4):279-283. Embrapa Agressilvipastoril, Rodovia MT 222, km 2,5, Caixa Postal 343, Sinop, MT 78.550-970, Brazil. E-mail: luciano.lopes@embrapa.br

This study aimed to evaluate the interference of tuberculin test on the gamma-interferon (INF γ) assay, to estimate the sensitivity and specificity of the INF γ assay in Brazilian conditions, and to simulate multiple testing using the comparative tuberculin test and the INF γ assay. Three hundred-fifty cattle from two TB-free and two TB-infected herds were submitted to the comparative tuberculin test and the INF γ assay. The comparative tuberculin test was performed using avian and bovine PPD. The INF γ assay was performed by the Bovigam™ kit (CSL Veterinary, Australia), according to the manufacturer's specifications. Sensitivity and specificity of the INF γ assay were assessed by a Bayesian latent class model. These diagnostic parameters were also estimate for multiple testing. The results of INF γ assay on D0 and D3 after the comparative tuberculin test were compared by the McNemar's test and kappa statistics. Results of mean optical density from INF γ assay on both days were similar. Sensitivity and specificity of the INF γ assay showed results varying (95% confidence intervals) from 72 to 100% and 74 to 100% respectively. Sensitivity of parallel testing was over 97.5%, while specificity of serial testing was over 99.7%. The INF γ assay proved to be a very useful diagnostic method.

INDEX TERMS: *Mycobacterium bovis*, gamma interferon, comparative tuberculin test, diagnosis.

RESUMO. [Estimação dos parâmetros e uso do teste do interferon gama para o diagnóstico da tuberculose bovina no Brasil.] O presente estudo avalia a interferência do teste de tuberculinização no teste do interferon gama

(INF γ), estima a sensibilidade e a especificidade do INF γ em condições brasileiras e simula a utilização dos testes múltiplos usando a tuberculinização comparada e o teste do INF γ . Trezentos e cinquenta animais oriundos de dois rebanhos livres e dois rebanhos positivos foram submetidos à tuberculinização comparada e ao teste de INF γ . A tuberculinização comparada foi realizada utilizando PPD aviária e bovina. O teste de INF γ foi realizado utilizando o kit Bovigam® (CSL Veterinary, Austrália) de acordo com as especificações do fabricante. A sensibilidade e especificidade do teste de INF γ foram calculadas pelo Modelo Bayesiano de Classe Latente. Esses parâmetros foram também estimados para os testes múltiplos. Os resultados do teste de INF γ no D0 e D3 após o teste de tuberculinização foram comparados pelos testes estatísticos de McNemar e kappa. Os resultados das médias de densidade ótica do teste de INF γ em ambos os dias foram similares. A sensibilidade e a especificidade do teste de INF γ apresentaram resultados variando (95% intervalo de confiança) de 72 a 100% e 74 a

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100%, respectivamente. A sensibilidade do teste em paralelo foi acima de 97,5% enquanto a especificidade do teste em série foi acima de 99,7%. O teste do $\text{INF}\gamma$ provou ser um método de diagnóstico muito útil.

TERMOS DE INDEXAÇÃO: *Mycobacterium bovis*, tuberculose bovina, interferon gama, tuberculinização intradérmica, diagnóstico, Brasil.

INTRODUCTION

Bovine tuberculosis is still a problem in Brazil. The only comprehensive survey, accomplished in Minas Gerais State in 1999, showed prevalences of infected herds and animals of 5.0% and 0.81% respectively (Belchior 2001). Currently other studies are in progress in some Brazilian States. Thus, in 2001, the *Ministério da Agricultura, Pecuária e Abastecimento* (MAPA) [Brazilian Ministry of Agriculture] started a national program for the control and eradication of brucellosis and tuberculosis (*Programa Nacional de Controle e Erradicação de Brucelose e Tuberculose* (PNCEBT), which among its policies uses a test-and-slaughter strategy (Brasil 2004). Bovine tuberculosis diagnosis is based on serial testing using caudal fold test, only for beef cattle, and single tuberculin test as screening tests and comparative tuberculin test for confirmatory test (Brasil 2006). However, the PNCEBT is prone to the inclusion of new tests and control strategies in the program, after its validation in Brazil.

In an attempt to detect *Mycobacterium bovis*-infected cattle as fast as possible, a simple and efficient laboratory method based on the measurement of gamma interferon ($\text{INF}\gamma$) produced by T lymphocytes of infected animals after *in vitro* antigenic stimulation was developed (Wood et al. 1990, Rothel et al. 1992, Wood & Jones 2001). This method showed many advantages compared to the tuberculin test, as just one visit to the farm is needed, repeated testing can be done at any interval because no antigen is inoculated into the animals, it can detect early infections, and its sensitivity is always considered to be similar or greater than that of tuberculin test (Wood et al. 2001, De La Rua-Domenech 2006). Its sensitivity varies from 73.0 to 100% and its specificity varies between 85.0 and 100% (Wood & Jones 2001, De La Rua-Domenech 2006). In Brazil, the sensitivity of the $\text{INF}\gamma$ assay was estimated to be 100% compared to the single tuberculin test (Lilenbaum et al. 1999) and to vary from 71.4 to 91.4%, while the specificity varied between 80.0 and 86.7%, relatively to the comparative tuberculin test at times from the inoculation of tuberculin to 21 days afterwards (Marassi et al. 2010). The use of tests as the tuberculin test that show less than perfect sensitivity and specificity (De La Rua-Domenech 2006, Marassi et al. 2010) to estimate the parameters of a new test can introduce bias in the estimated parameters (Enøe et al. 2006, Dendukuri et al. 2009). Moreover, the $\text{INF}\gamma$ assay showed decrease rates of sensitivity and specificity with increasing time after tuberculin inoculation (Monaghan et al. 1994), but the effect of recent tuberculin test on the performance of the $\text{INF}\gamma$ assay is very conflicting on the literature (Rothel et al. 1992, Whipple et al. 2001, Rangen et al. 2009), probably due to the type of tuberculin test used and testing conditions.

Thus, the aims of present study were (i) to evaluate the interference of tuberculin test on the $\text{INF}\gamma$ assay, (ii) to estimate the sensitivity and specificity of the $\text{INF}\gamma$ assay in Brazilian conditions, and (iii) to simulate multiple testing using the comparative tuberculin test and the $\text{INF}\gamma$ assay in a control and eradication program for bovine tuberculosis (TB).

MATERIALS AND METHODS

Animals. The three hundred-fifty adult dairy Holstein-Zebu crossbred cattle submitted to the comparative tuberculin test in the study were from four herds in Minas Gerais State, Brazil: two TB-free and two TB-infected herds. The TB-infected herds were selected previously according historical comparative tuberculin test positive results, which showed macroscopic lesions at necropsy and isolation of *Mycobacterium bovis* from lesions. The TB-free herds were selected previously according historical comparative tuberculin test negative results. Skin test-positive animals from TB-infected herds were enrolled in the present study as infected animals. Skin test-negative animals from TB-free herds were enrolled as TB-negative animals. Skin test-inconclusive animals as well as negative reactions, from any herd, were not enrolled in the study.

Comparative tuberculin test. The comparative tuberculin test was performed according to PNCEBT [2, 3] using avian and bovine PPD (*Ministério da Agricultura, Pecuária e Abastecimento* - MAPA, Brazil). Skin thickness at injection site was measured before PPD injections and at 72 ± 6 h post-inoculation by the same person using the same calipers.

Blood samples. Blood samples were collected through jugular venopuncture into heparinized vacuum tubes (Sarstedt, Germany) in two occasions: (i) immediately before the inoculation of both PPDs (D0) and (ii) three days later, during the reading of comparative tuberculin test (D3). Samples were transported at room temperature to the laboratory and processed within 24 hours from collecting for $\text{INF}\gamma$ assay.

$\text{INF}\gamma$ assay. The $\text{INF}\gamma$ assay was performed by the Bovigam™ kit (CSL Veterinary, Australia), according to the manufacturer's specifications. Briefly, each blood sample was divided in three aliquots of 1.5mL, which were distributed into wells of 24-well plates (Sarstedt, Germany). Then, 100 μL of bovine PPD (CSL Veterinary, Australia), avian PPD (CSL Veterinary, Australia), or PBS (pH 7.3) were added to one of the wells. Thereafter, plates were incubated at 37°C, in humidified 5% CO_2 atmosphere, for 24 hours as described by (Rothel et al. 1992). After incubation, plates were centrifuged at 500 x G for 10 min at 4°C, and 500 μL of plasma from each well were collected, identified and stored 123 at -20°C until assayed, in duplicate, by the $\text{INF}\gamma$ Bovigam™ ELISA.

Statistical analysis. The results of $\text{INF}\gamma$ assay on D0 and D3 after tuberculin inoculation and the comparative tuberculin test were compared by the McNemar's test (Siegel 1975) and kappa statistics (Smith 1994). The mean optical density of the results from bovine PPD stimulated blood in the $\text{INF}\gamma$ assay on D0 and D3 was compared by the Wilcoxon test (Siegel 1975). Sensitivity and specificity of tests were calculated by a Bayesian latent class model (Dendukuri & Joseph 2001). Prior distributions for the sensitivity and specificity for comparative tuberculin test (Rothel et al. 1992, Marassi et al. 2010) and $\text{INF}\gamma$ assay (Wood & Jones 2001) were specified from the literature. Parameters for parallel and serial testing were calculated according to Tarabla (Tarabla 2000). Statistical analyses were performed by the statistical packages Epistat (T.L.Gustafson, Round Rock, Texas, USA), WinEpiscope 2.0 (Thrusfield et al. 2001), and BayesLatentClassModels 1.0 (N.

Dendukuri, P. Bélisle, and L. Joseph, McGill University, Montreal, Canada), with an α error of 0.05 (Sampaio 2002).

RESULTS

From the 350 cattle submitted to the comparative tuberculin test, 102 were selected to the study: 29 comparative tuberculin test-positive animals from TB-infected herds and 73 comparative tuberculin test-negative animals from TB-free herds. Among the comparative tuberculin test-negative animals, 39 cattle showed cross-reactivity with *Mycobacterium avium* complex in the comparative tuberculin test (data not shown). Results for the comparative tuberculin test and INF γ assay in all 102 animals on D0 and D3 are shown in Table 1.

Table 1. Results of the Comparative Tuberculin Test and INF Assay performed on blood collected on day 0, before the inoculation of tuberculin, and on day 3, at comparative tuberculin test reading, in cattle from tuberculosis-positive and tuberculosis-free herds

IFN γ Assay	Comparative Tuberculin Test		
	Positive	Negative	Total
D0*			
Positive	24	16	40
Negative	5	57	62
Total	29	73	102
D3**			
Positive	25	11	36
Negative	4	62	66
Total	29	73	102

* D0 = Test performed on blood collected on day 0, before the inoculation of tuberculin. χ^2 McNemar = 4.761, P = 0.029, df = 1. ** D3 = Test performed on blood collected at comparative tuberculin test reading on day 3. χ^2 McNemar = 2.4, P = 0.118, 340 df = 1.

There was a significant difference between the performance of the comparative tuberculin test and the INF assay done on D0, however, no difference was found when the results from INF γ assay done on D3 were compared (Table 1). The agreement between the two methods was 79.4% ($\kappa=0.546$) on D0 and 85.3% ($\kappa=0.663$) on D3. If data from animals with cross-reactivity with *M. avium* complex were withdrawn from the analyses, the agreement among the methods rises to 82.5% ($\kappa = 0.649$) on D0 and 85.7% ($\kappa=0.713$) on D3.

No significant differences were found on the mean optical density of the results from bovine PPD stimulated

blood in the INF γ assay on D0 (mean 1.1671) and D3 (mean 0.9482) (P=0.0646).

The estimates of sensitivity and specificity for comparative tuberculin test and INF γ assay on D0 and on D3 are shown in Table 2. Parameter estimates for multiple testing performed with comparative tuberculin test and INF γ assay are shown in Table 3.

Table 3. Estimates of Sensitivity and Specificity of multiple testing performed with Comparative Tuberculin Test and INF γ Assay

Day	Parallel Testing (%)		Serial Testing (%)	
	Sensitivity	Specificity	Sensitivity	Specificity
D0 *	97.5	90.0	68.5	99.8
D3 **	98.2	86.3	72.5	99.7

* D0 = Test performed on blood collected on day 0, before the inoculation of tuberculin. ** D3 = Test performed on blood collected at comparative tuberculin test reading on day 3.

DISCUSSION

The results of the present study confirm the usefulness of the INF γ assay for the diagnosis of bovine tuberculosis and shows that it could be a very useful tool in the context of a control and eradication program, in conjunction with the comparative tuberculin test. On the other hand, INF γ assay has some limitations regarding the structure of laboratory and laboratory technicians required for the exams. Another downside of INF γ assay cannot be ruled out is the cost of commercial kits, much higher than skin test. However, despite those limitations, the INF γ assay can be used from a strategic standpoint. Simulation of its use in multiple testing with the comparative tuberculin test demonstrates that it could help to solve some of the most complicated and strategic situations on a bovine TB-control and eradication program. One of the greatest drawbacks in bovine TB diagnosis is lack of sensitivity of its international standard, the tuberculin test (De La Rua-Domenech et al. 2006, Monaghan et al. 1994, OIE 2009). Most figures showed values around 80% sensitivity for comparative tuberculin test, similar with the estimations found in this study (Table 2), which precludes a rapid evolution of a control program. The use of parallel testing with the INF γ assay and the comparative tuberculin test showed much higher rates of sensitivity, as shown by the simulation performed (Table 3). Hence, this strategy could be very useful in the beginning of a control program or for the introduction of animals into free-herds or free-regions, when a very high sensitivity is needed for the diagnosis to increase the negative predictive

Table 2. Estimates of sensitivity and specificity for the Comparative Tuberculin Test and the INF γ Assay on the day of inoculation (D0) and on the day of reading (D3) of tuberculin test*

Day	Comparative Tuberculin Test						INF γ Assay					
	Sensitivity (%)			Specificity (%)			Sensitivity (%)			Specificity (%)		
	Median	95% CI **		Median	95% CI		Median	95% CI		Median	95% CI	
D0 ***	76.7	59.7	93.7	97.8	95.7	99.0	89.3	71.7	100	92.0	73.7	100
D3 ****	79.4	60.9	93.8	97.7	95.5	98.9	91.3	72.8	100	88.3	73.4	100

* All data were calculated by a Bayesian latent class model (Dendukuri & Joseph 2001), with prior distributions from the literature (Monaghan et al. 1994, Wood & Jones 2001). ** 95% CI = 95% Credible Interval. *** D0 = Test performed on blood collected on day 0, before the inoculation of tuberculin. **** D3 = Test performed on blood collected at comparative tuberculin test reading on day 3.

value and to reduce the number of false-negative animals, which could introduce the bovine tuberculosis into a new area (Snider 1982, Neill et al. 1994, Whipple et al. 1995, Archetti et al. 1996, Llamazares et al. 1999, Cagiola et al. 2004, Vordermeier et al. 2004).

The use of the INF γ assay as a confirmatory test is also advisable by the simulations done, which showed a very high specificity rate when serial testing with the two assays were used (Table 3). Most programs on the control and eradication of bovine TB are based on serial testing with single tuberculin test followed by a confirmatory comparative tuberculin test (Brasil 2006, OIE 2009). If the INF γ assay is added to this strategy, as another confirmatory test in the series, a nearly 100% specificity will be achieved, which is essential in the latest phases of an eradication program or in free-areas to increase the positive predictive value of the diagnosis (Smith 1994, Tarabla 2000).

The sensitivity and specificity values estimated for the INF γ assay and for the comparative tuberculin test in the present study are in the range of those reported for these assays in the literature (Monaghan et al. 1994, Wood & Jones 2001, De La Rua-Domenech et al. 2006). The specificity of the comparative tuberculin test calculated here agrees with previous reports from Brazil (Belchior 2001) that estimated it to be over 95%. Nevertheless, the sensitivity and specificity estimates for the INF γ assay showed rates less than that desired for a test to be employed as the sole test in a control and eradication program (Table 2), therefore discouraging its use as a single test in the diagnosis of bovine TB. Previous reports from Brazil found different mean figures for sensitivity and specificity of the INF γ assay (Lilenbaum et al. 1999, Marassi et al. 2010) although they are in the same range as the credible interval found in the present study. The way these parameters were calculated on those studies, relatively to the single or comparative cervical tuberculin test, could have accounted for the differences. In contrast, the Bayesian latent class model use in this study prevents the use of a gold standard with imperfect sensitivity and specificity to which all values were relatively calculated (Dendukuri & Joseph 2001, Dendukuri et al. 2009). Thus, more confident parameters could be estimate, with the advantage of enabling the calculation of credible intervals for the parameters.

An anamnestic response to the comparative tuberculin test was not detect by the INF γ assay in the present study as no difference was found in the mean optical density values of INF γ assay performed on blood samples before the inoculation of PPD and at tuberculin reading, even though it has been observed in some studies (Rothel et al. 1992, Wood & Jones 2001). Nevertheless, it has not hindered INF γ assay as the major analyses showed no difference between tests performed on samples collected on both days. Thus, the present results of INF γ assay performed on blood samples collected from cattle tuberculin-tested three days previously showed that the assay was not affected by recent tuberculin test, confirming recent studies on this conflicting subject (Whipple et al. 1995, Rangen et al. 2009).

One of the major concerns for the use of the INF γ assay in large countries, as Brazil, is the time interval from sample

collection to laboratory processing of material. Former studies indicate that a strict interval around 8h from sampling to laboratory analysis should be used (Rothel et al. 1992, Wood & Jones 2001). This would preclude its use in areas far from a laboratory, as is the case of the greatest beef producing area in Brazilian Midwest, but the present results showed that even if blood samples were processed within 24h from collection, the INF γ assay is a very useful test, as suggested by other studies (Ryan et al. 2000, Buddle et al. 2001).

Thus, the INF γ assay proved to be a very useful diagnostic method to be incorporate in a control and eradication program for bovine tuberculosis, due to the adaptability of its use to different epidemiological situations. Moreover, the parameters studied here demonstrated that the INF γ assay is applicable to frequently tuberculin-tested cattle in areas where blood samples could be processed within 24h from collection.

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