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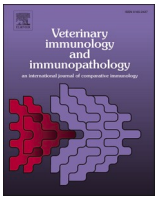

Performance of two commercial serological assays for bovine tuberculosis using plasma samples

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ABSTRACT

In the bovine tuberculosis diagnosis, the use of plasma samples (already available for IFNɣ assays) in serological tests might facilitate the work in the field. Here, the performance of two commercial serological tests (ELISA IDEXX *M. bovis* Ab test and Enferplex Bovine TB antibody test) were evaluated using plasma samples from cattle in Belgium. Specificity values estimated from 567 plasma samples collected from bTB-free cattle were 98.4% when using the ELISA IDEXX *M. bovis* Ab test, and were 96.5% and 93.3% when using the high specificity and high sensitivity settings of the Enferplex Bovine TB antibody test, respectively. Sensitivity values were calculated relative to SICCT-positive (N = 117) and IFNɣ-positive (N = 132) animals originating from *M. bovis*-infected herds. Overall, the multiplexed Enferplex Bovine TB antibody test had better sensitivity (mean: 32.5% and 43.4% for the high specificity and sensitivity settings, respectively) compared to the ELISA IDEXX *M. bovis* Ab test (mean: 12%). Data obtained from plasma samples in the current study were compared to a previous study using both serological tests with sera. In conclusion, both serological tests showed comparable performance with both matrix; although overall specificity values with the Enferplex Bovine TB antibody test were lower when using plasma samples than sera.

1. Introduction

Bovine tuberculosis (bTB), caused by the pathogen *Mycobacterium bovis* (*M. bovis*) remains a major economic problem in many developed countries. In Europe, surveillance programs are implemented to control the disease and document its status across countries. However, despite Belgium having an officially tuberculosis free (OTF) status, conferred by the European commission in 2003, it is still subject to a few sporadic bTB-outbreaks each year (https://www.favv-afsca.be/santeanimale/tuberculose/). The complexity of the immune response of cattle to *M. bovis*, as well as the specific characteristics of the pathogen, results in animals expressing different stages of the infection. This phenomenon limits the performance of current diagnostic tests, and might contribute to the persistence of the disease (Bezos et al., 2014; Cassidy, 2006; de la Rua-Domenech et al., 2006).

Serological tests are easy to implement, and allow a large range of sampling approaches to be used (serum, plasma and/or milk). Thus, these tests could be used to improve bTB diagnosis. However, several studies have reported that the antibody response of cattle to *M. bovis* is not uniform; consequently, serological tests using multiple antigens at once could improve the detection of this disease in animals at different stages of infection (Amadori et al., 2002; Fifis et al., 1992; Lyashchenko et al., 2017). Several platforms of multiplexing have been developed over the last two decades; these include Luminex, Meso scale Discovery (MSD), and Enferplex (Chowdhury et al., 2009; O'Brien et al., 2023; Whelan et al., 2008). However, to date, the Enferplex Bovine TB antibody kit is the only multiplexed commercial kit to detect bTB in cattle.

As serology and interferon gamma (IFNɣ) tests can be performed concurrently during surveillance programs, plasma (already collected for IFNɣ assays) could be used in serological tests. This approach would facilitate the fieldwork of veterinarians, with just one sample being required per animal. Currently, only the ELISA IDEXX *M. bovis* Ab test (not the multiplexed Enferplex Bovine TB antibody test) is described for use with both serum and plasma samples (OIE, 2019, 2012). Here, we present the first evaluation of the commercial Enferplex Bovine TB Antibody kit using plasma samples from cattle, in comparison with

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commercial ELISA IDEXX *M. bovis* Ab test.

2. Material and methods

A total of 567 bovine plasma samples were collected from animals originating from 27 bTB-free herds in Belgium (OTF country) that had negative results with the IFNɣ assay. The delay between the last skin test and the blood sampling was not known for samples from negative animals. Therefore, it was not possible to determine if samples were taken within the amnestic window (i.e. 5–30 days post-tuberculin injection). These samples were used to estimate the diagnostic specificity (DSp) of serological assays. Plasma samples, used for the estimation of the relative sensitivity (RSe), were collected (2016–2018) from three bTB-outbreaks in cattle herds at 15–30 days post skin test. One-hundred and seventeen plasma samples were collected from animals having positive results (bovine-PPD 4 mm > avian-PPD) or inconclusive results (bovine-PPD 1–4 mm > avian-PPD) in the single intradermal cervical comparative tuberculin (SICCT) test. For the purpose of this study, SICCT-doubtful results were considered positive, as all tested animals originated from *M. bovis*-infected herds. One hundred and thirty-two plasmas samples were collected from animals with positive results in the IFNɣ assay (tests performed with ID Screen® Ruminant IFN-g from Innovative Diagnostics, a cut off of 35% was used as indicated by the manufacturer). For all plasma samples assessed serologically in this work, blood samples were collected in lithium heparin tubes, centrifuged and plasma samples were then stored at − 20 °C until to be used. Ethical approval to collect blood samples was not required for this study. Plasma samples, SICCT and IFNɣ results were obtained within the framework of the national Belgian bTB control program, in compliance with official guidelines from the Federal Authority for the control of the bovine TB in Belgium (i.e., Federal Agency for the Safety of the Food Chain [FASFC] and veterinary services). The ELISA IDEXX *M. bovis* Ab test (IDEXX Laboratories, Westbrook, ME, USA), called ELISA IDEXX hereafter, was performed as indicated by the manufacturer. A positive result was defined as a Sample/Positive (S/P) ratio ≥ 0.30. The Enferplex Bovine TB antibody test (Enfer Scientific ULC, Naas, co. Kildare, Ireland), called Enfer11Ag TB hereafter, contained 11 antigens (nature of antigens not disclosed by EnferGroup) presented under form of spots in each well along with a blank (i.e. a spot without antigen tested with samples). This test was performed with plasma samples as indicated by the manufacturer for sera and described in details by Moens et al., (2023). In brief, after incubation of antigens with samples and the conjugate, antigen-antibody reactions were highlighted by the addition of the chemiluminescent substrate. Signals, emitted by each spots and expressed as relative light units (RLU), were captured by Q-view™ imagery system (Quansys Biosciences, Logan, USA). The results were interpreted according to the high specificity (HSP) and high sensitivity (HSE) interpretation settings of the kit, using the two-positive antigens rule. A previous study performed by our team (Moens et al., 2023), evaluated the performance of the Enfer11Ag TB with sera ($N = 641$) from different animals in Belgian herds. Blank values obtained with bovine sera in this previous study were compared to blank values obtained using bovine plasma samples in the current study in order to determine if there is different backgrounds inherent to the matrix used. The Mann-Whitney test was used to calculate the difference between blank values from bovine serum and plasma samples. The degree of agreement between tests was determined using the kappa (k) coefficient. A contingency 2 × 2 table based on the Chi-square test (1 degree of freedom) was used to determine any differences between tests depending on the method and type of sample. In all statistical tests, performed with the GrapPadPrism statistical tool, a P-value (p) < 0.05 was considered statistically significant. The sensitivity and the specificity of tests were estimated using a binomial exact.

3. Results and discussion

The DSp value obtained with ELISA IDEXX and Enfer11Ag TB tests are shown in Table 1. The DSp value obtained with the ELISA IDEXX test when using plasma samples was significantly higher compared to the DSp values with the Enfer11Ag TB test (ELISA IDEXX versus Enfer11Ag TB (HSP): $p = 0.04$; ELISA IDEXX versus Enfer11Ag TB (HSE): $p < 0.0001$). Plasma samples used here seemed to grip onto the wells more than sera used by Moens et al., (2023) in the Enfer11Ag TB test, which could increase nonspecifically their RLU values. Accordingly, blank values were significantly higher ($p < 0.0001$) for plasma samples compared to serum samples (Fig. 1). White et al. (2013) suggested that the presence of clotting factors in plasma might increase the adhesive properties of samples, generating a higher background that was likely to reduce the specificity of serological diagnostic tests. Previous studies showed that the anti-coagulant used in the blood collecting tubes may also affect the antibodies measure (Biancotto et al., 2012; Brøndum et al., 2016) and might thus affect assays results, in particular results from multiplexed assays, obtained with plasmas samples compared to serum samples, with animals misclassified as being positive or negative. Moreover, the use of more antigens in the multiplexed test might increase the risk of cross-reactions with proteins from environmental mycobacteria that are potentially present in cattle (Biet and Boschiroli, 2014; Gcebe et al., 2016; Varela-Castro et al., 2022; Waters et al., 2011), impacting the specificity of the Enfer11Ag TB test.

When comparing specificity values estimated in this work using plasma samples with specificity values reported by Moens et al., (2023) using sera from different population of bTB-free cattle, with ELISA IDEXX and Enfer11Ag TB no significant difference in DSp values was observed (Table 1). Thus, our results suggest that the use of plasma compared to sera has no, or limited, impact on specificity results in the two serological tests. The slight differences observed between the two studies might be attributed to difference of the population of cattle that was tested and the number of tested samples. This finding is consistent with previously published work, which supported that the ELISA IDEXX and Enferplex methods appeared to perform adequately using plasma or serum samples (Casal et al., 2014; McCallan et al., 2021; Waters et al., 2011); however, it should be noted that the Enfer11Ag TB kit used in the current study is a modified version of those used in previous studies with four or six antigens.

For both methods used in the present study (ELISA IDEXX and Enfer11Ag TB kits), sensitivity values were calculated relative to SICCT-positive animals and IFNɣ-positive animals (Table 2). As previously reported with sera (Moens et al., 2023), the sensitivity values were significantly higher for the multiplexed Enfer11Ag TB test ($p < 0.0001$) compared to the ELISA IDEXX test using the same plasma samples. The ELISA IDEXX and Enfer11Ag TB HSP (k = 0.17) values slightly agreed, as well as the ELISA IDEXX and Enfer11Ag TB HSE values (k = 0.14).

Table 1
Diagnostic specificity (DSp) values for ELISA IDEXX and Enfer11Ag TB tests using plasma samples (current study) and serum samples (Moens et al., 2023) from bTB-free cattle. Chi-square was performed to evaluate statistical difference between values reported by both studies.

	ELISA IDEXX	Enfer11Ag TB HSP	Enfer11Ag TB HSE
Current study data [Plasma samples]	N = 567 DSp: 98.4% (95% CI: 97.4–99.4)	N = 567 DSp: 96.5% (95% CI: 95.0–98.0)	N = 567 DSp: 93.3% (95% CI: 91.2–95.4)
Moens et al. (2023) data [Serum samples]	N = 308 DSp: 97.1% (95% CI: 94.5–98.7)	N = 308 DSp: 97.1% (95% CI: 94.5–98.7)	N = 172 DSp: 95.1% (95% CI: 92.1–97.3)
p-value (Chi2 test)	0.18	0.63	0.28

HSP: High specificity setting; HSE: High sensitivity setting; CI: Confidence interval

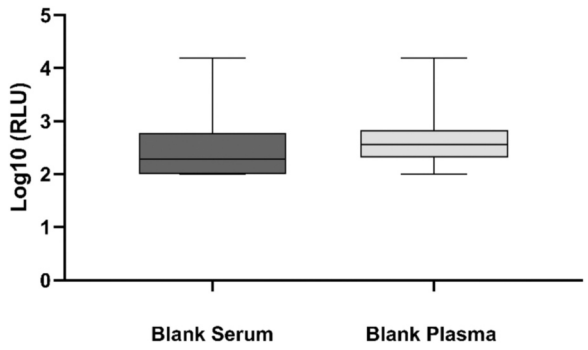

Fig. 1. Difference between values of relative luminescence units (RLU) measured in the Enfer11Ag TB test for the blank from serum ($N = 641$; dark gray) and plasma ($N = 837$; light gray) samples tested. RLU values are expressed in Log10. Values of blank for plasma samples were statistically higher than values of blank for sera ($p < 0.0001$; Mann-Withney test).

Table 2
Sensitivity values estimated for the ELISA IDEXX and Enfer11Ag TB tests relative to data from the single intradermal cervical comparative tuberculin (SICCT) test and the interferon gamma (IFNɣ) assay.

Serological test	Comparator test	Sensitivity	95 CI %
ELISA IDEXX	SICCT (N = 117)	10.3% (12/117)	4.8–15.8
Enfer11Ag TB HSP	SICCT (N = 117)	31.6% (37/117)	23.2–40.1
Enfer11Ag TB HSE	SICCT (N = 117)	43.6% (51/117)	34.6–52.6
ELISA IDEXX	IFNɣ (N = 132)	13.6% (18/132)	7.8–19.5
Enfer11Ag TB HSP	IFNɣ (N = 132)	33.3% (44/132)	25.3–41.4
Enfer11Ag TB HSE	IFNɣ (N = 132)	43.2% (57/132)	34.7–51.6

HSP: High specificity setting; HSE: High sensitivity setting; CI: Confidence interval

Aside from the technological and interpretational (S/P versus two-positive antigens rule) differences of the two methods, differences in sensitivity (and weak agreement) might be attributed to the number of antigens (two versus 11 antigens in ELISA IDEXX versus Enfer11Ag TB, respectively).

Overall, the sensitivity values (versus SICCT) obtained with the ELISA IDEXX and Enfer11Ag TB tests using plasma samples were significantly lower ($p < 0.05$) than values reported with sera (Moens et al., 2023) from SICCT-positive cattle (Table 3). However, comparison of the sensitivity results obtained in the two studies was of limited value, because different populations of bTB-positive animals were used. Accordingly, stages of infection and antibody response kinetics might be different. Two previous studies evaluated the performance of the ELISA IDEXX and Enferplex methods (four and six antigens, respectively) using bovine plasma samples (Casal et al., 2014; McCallan et al., 2021). The mean values of sensitivity reported by McCallan et al. (2021) were 7.4% for ELISA IDEXX and 4.9% for Enferplex TB, whereas these values were 68.5% and 84.3%, respectively, in (Casal et al., 2014). These differences could be attributed to several reasons, including the cattle population used, study design (sampling prior/post skin test), results interpretation settings, the cut-off values and number of antigens used in Enferplex tests. Of note, plasma samples from animals used in this study along with sera used by Moens et al., (2023) to determine the sensitivity values of both serological assays were collected within the anamnestic window, beneficing of "boost" effect of the humoral response (Casal et al., 2014). It should thus be expected that RSe of serological assays evaluated in this work are decreased when using blood sampling from non-boosted animals.

The profiles of recognized antigens by the antibodies of bovine plasma samples originating from bTB-free herds and bTB-positive herds, and deemed "positive" according to the Enfer11Ag TB test (HSP setting), were analyzed. As previously observed with sera (Moens et al., 2023),

Table 3
Comparison of sensitivity values estimated with ELISA IDEXX and Enfer11Ag TB tests relative to positive animals with the single intradermal cervical comparative tuberculin (SICCT) test using plasma samples (current study) and serum samples (Moens et al.,2023). Chi-square was performed to evaluate statistical difference between values reported by both studies.

	ELISA IDEXX	Enfer11Ag TB HSP	Enfer11Ag TB HSE
Current study data [Plasma samples]	N = 117 RSe (vs SICCT): 10.3% (95% CI: 4.8–15.8)	N = 117 RSe (vs SICCT): 31.6% (95% CI: 23.2–40.1)	N = 117 RSe (vs SICCT): 43.6% (95% CI: 34.6–52.6)
Moens et al. (2023) data [Serum samples]	N = 172 RSe (vs SICCT): 36.6% (95% CI: 29.4–43.8)	N = 172 RSe (vs SICCT): 51.7% (95% CI: 44.3–59.2)	N = 172 RSe (vs SICCT): 58.7% (95% CI: 51.4–66.1)
p-value (Chi2 test)	0.0001	0.0007	0.01

HSP: High specificity setting; HSE: High sensitivity setting; RSe: relative sensitivity; CI: Confidence interval

the first five antigens were mainly represented (under different associations) in the positive population. These antigens were assumed to include the sero-dominant MBP70 and MPB83 under peptide and/or recombinant form (Casal et al., 2014; Moens et al., 2023; Whelan et al., 2010, 2008). In addition, the association of the antigens 1 and 4 was the main profile found in the positive population when compared to the negative population. In contrast, only two profiles were present in the negative population, and only antigens 6–11 were implicated. These results suggest that the main profiles of antigens previously identified in positive and negative herds of cattle are not modified according to the matrix used.

The current study showed that the specificity values calculated when using plasma samples with the ELISA IDEXX and Enfer11Ag TB tests were not significantly different from previous data using sera (Moens et al., 2023), although lower with the Enfer11Ag TB test. Thus, preliminary data obtained suggest that plasma samples might be used like sera in the bTB diagnosis with both serological assays under study. However, optimizing of the Enfer11Ag TB when using plasma samples might be necessary to reach a similar specificity of that of the ELISA IDEXX. As the use of plasma samples was not included in the protocol established by Enfergroup for the Enfer11Ag TB assay, in contrary to the ELISA IDEXX, the same dilution (1:200 ratio) was used for plasma and serum samples. Therefore, new experiments with variable plasma and/or conjugate dilutions should be performed to optimize the use of the Enfer11Ag TB. Moreover, additional studies using plasma and serum samples from same negative and positive cattle are needed to confirm the data obtained in this work. It is however important to note that the serological assays fit within the bigger picture of bTB testing. As a consequence, their application and performance may vary according to multiple factors such as the associated CMI-based test (IFNg assay and/or SICCT test), the slaughterhouse surveillance, the geographic area, the bTB herd prevalence or the surveillance program applied (e.g. global screening, risk-based survey).

Declaration of Competing Interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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