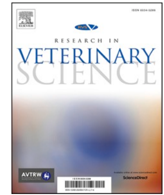
Research in Veterinary Science 159 (2023) 125–132

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc



Field evaluation of two commercial serological assays for detecting bovine tuberculosis

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ARTICLE INFO

Keywords:
Bovine tuberculosis
Enferplex bovine TB
ELISA IDEXX
Surveillance program
Multiplexed serological assay
Serial interpretation

ABSTRACT

Diagnosis of bovine tuberculosis in cattle is challenging due to complex immune host response to infection that limit the performance of available diagnostic tests. In this study, performance of two commercial serological assays developed to detect bovine tuberculosis were evaluated: Enferplex Bovine TB antibody kit including 11 antigens (EnferGroup, Ireland) and IDEXX *M. bovis* Ab kit (IDEXX, USA). The specificity value obtained with the ELISA IDEXX *M. bovis* Ab test was 97.1%, whereas it was 97.1% and 95.1% for the high specificity and sensitivity settings, respectively, with the Enferplex Bovine TB antibody kit. The sensitivity of the multiplexed Enferplex Bovine TB antibody test for SICCT-positive animals was higher (N = 172; 51.7% and 58.7% with high specificity and sensitivity settings, respectively) compared to the ELISA IDEXX *M. bovis* Ab test (sensitivity of 36.6%). "Antigen profiles" generated by the multiplexed Enferplex method showed that five out of 11 antigens present in the test were mostly identified as positive sera in cattle originating from bTB-outbreaks. In comparison, unique profiles appeared to be correlated with false positive results. However additional studies are needed to confirm the observed antigen profiles, and their potential use as an additional diagnostic tool. Serial interpretation of the two serological tests produced higher diagnostic specificity (>99%), reducing false positive results, which is essential for a screening test when the prevalence of bovine tuberculosis is low.

1. Introduction

Bovine tuberculosis (bTB) is a zoonotic disease that is mainly caused by *Mycobacterium bovis,* a member of the *Mycobacterium tuberculosis* complex. Although *M. bovis* infects a wide range of host species, the primary animal reservoir is cattle. Even though the impact of bTB on the public health is limited in Europe (due to existing mitigation measures, e.g. pasteurization) (EFSA, 2013), it remains a major economic problem due to reduction in production, limitation of trade and slaughter of infected animals (Caminiti, 2019; Pérez-Morote et al., 2020).

National surveillance programs are mandatory for each country in Europe and in 2003, Belgium obtained the officially tuberculosis free (OTF) status by the European commission (EC Decision 2003/467/EC). Yet, the country is not actually biologically free of bTB (herd prevalence <0.1% annually), with sporadic outbreaks occurring each year. Specifically, five, six, one, and five bTB outbreaks were reported in 2017, 2018, 2020, and 2021, respectively (https://www.favv-afsca.be/santeanimale/tuberculose). Thus, an efficient surveillance program is required to maintain and document the OTF status.

Until 2021, the national tuberculosis surveillance program in Belgium, based on the Council Directive 64/432/EEC, had two main axes: (i) periodic bTB screening of cattle with Single Intradermal Comparative Cervical Tuberculin (SICCT) test; and (ii) systematic examination of post mortem tuberculous lesions at the slaughterhouse. However, in 2016, a scientific report issued from the Federal Agency for the Safety of the Food Chain (FASFC) highlighted the difficulties of implementing of the SICCT test in the field, and the need to set up new diagnostic tests with high specificity (Federal Agency for the Safety of the Food Chain (FASFC), 2016). Accordingly, a task force, composed of various animal health stakeholders, was established to investigate possible alternatives (Welby et al., 2022). The use of the serological assays was considered as one possible alternative.

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https://doi.org/10.1016/j.rvsc.2023.04.004
Received 6 February 2023; Received in revised form 3 April 2023; Accepted 11 April 2023
Available online 12 April 2023
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Cell-mediated immunity (CMI) is predominant during the early and intermediate stages of the infection, driven by Th1 lymphocytes. As the disease progress, the response of Th1 lymphocytes is progressively replaced by that of Th2 lymphocytes. This phenomenon is associated with a lower CMI-based response, and the development of a humoral immune response (Pollock and Neill, 2002; Welsh et al., 2005). Consequently, antibody-based assays were considered mainly for identifying animals with chronic infection that were missed by standard CMI-based tests (Waters et al., 2017). Although this "immune scenario" exists, the immune response of *M. bovis* infected animals might be more complex that initially thought, with the humoral response being a potential target for diagnostic tests. Over the last 20 years, methods have shown that antibodies can also be produced at different stage of infection, including early stages, and could be diagnostically useful (Amadori et al., 2002; Lyashchenko et al., 2017b; Mcnair et al., 2001; Waters et al., 2006).

Serological tests used to detect bovine tuberculosis are still generally based on the classical ELISA method. However, this method uses a limited number of antigens. The kinetics of the antibody response to *M. bovis* antigens noticeably varies during infection and none of the antigens discovered to date can be used to detect all animals infected with it at all stages of infection (Amadori et al., 2002; Lyashchenko et al., 2017a). Therefore, the use of serological tests to analyze a sample with multiple antigens could prove highly useful. The Enferplex TB method including 11 antigens, developed by the EnferGroup company, is currently the only commercial multiplexed assay available. Other methods based on the same technology developed by EnferGroup were previously published, but included a different number of antigens, and were not commercialized as kits (Casal et al., 2014; McCallan et al., 2021; Whelan et al., 2010).

Here, we aimed to provide the first evaluation of the commercial Enferplex Bovine TB Antibody kit including 11 antigens in cattle populations in Belgium, and to compare its performance with the commercial ELISA IDEXX *M. bovis* Ab kit.

2. Material and methods

2.1. Ethical approval

Ethical approval to collect blood samples was not required for this study. Serum samples, organ samples for bacteriology, and SICCT 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).

2.2. Serum samples

In total, 308 bTB-negative sera were obtained from cattle herds in Belgium (Belgian bTB-prevalence <0.1%), with a known history of being *M. bovis*-free for at least 5 years according to measures established by the Council Directive 64/432/EEC. The delay between the last skin test and the blood sampling was not known for samples from negative animals. Thus, it was not possible to determine if samples were taken within the amnestic window (i.e. 5-30 days post-tuberculin injection). An additional 333 serum samples were collected between 2016 and 2020 from four cattle herds in Belgium with natural *M. bovis* infection confirmed by bacterial isolation or RT-PCR (i.e., bTB-outbreaks). These data were analyzed as part of the bTB diagnosis by the Sciensano veterinary bacteriology service, the Belgian reference laboratory for bTB diagnostic. Out of these 333 bovine sera, 161 and 172 sera originated from animals with SICCT-negative results and positive or doubtful SICCT results, respectively. For the purposes of this study, "doubtful" SICCT results were considered positive, as all tested animals originated from *M. bovis*-infected herds. Blood was sampled 15–30 days after the diagnostic skin test. From the four bTB-outbreaks, 126 organs were collected and used in culture and RT-PCR to determine the presence of *M. bovis*. Accordingly, 38 animals were found to be positive in culture and/or RT-PCR (bacteriology-positive animals), and 65.8% (25/38) had gross lesions.

2.3. IDEXX M. bovis Ab test

The IDEXX *M. bovis* Ab test (termed ELISA IDEXX) was used following the manufacturer's instructions (IDEXX Laboratories, Westbrook, ME, USA). In brief, serum samples and controls were diluted at a 1:50 ratio, and were incubated for 60 min (±5 min) at 22 ± 4 °C. After a washing step, the conjugate was added, and the plate was incubated for 30 min (±2 min) at 22 ± 4 °C. The process was carried out by adding substrate solution and was stopped after 15 min (±1 min) at 22 ± 4 °C in the dark. Plates were read at 450 nm using a standard spectrophotometer. The data were analyzed following the manufacturer's instructions. A positive result was defined as a sample/positive (S/P) ratio ≥ 0.30, and a negative result was defined as an S/P ratio < 0.30.

2.4. Enferplex Bovine TB antibody test

The Enferplex Bovine TB antibody test (hereinafter Enfer11Ag TB) is produced by the EnferGroup company (Enfer Scientific ULC, Naas, co. Kildare, Ireland). It uses 11 antigens as an individual spot within a single well, and was used as described by the manufacturer. The company has not publicly disclosed the nature of antigens used in the kit. In brief, serum samples and controls were diluted to a 1:200 ratio. They were then incubated and shaken for 60 min at 37 ± 2 °C. After a washing step, the conjugate was added, and the plate was incubated and shaken at 37 ± 2 °C for 60 min. Signals as relative light units (RLU) were captured with a Q-View™ Imager LS immediately after the substrate was added. The data were analyzed using Q-View™ Software (Quansys Biosciences, Logan, USA). The index of response of an antigen (Ag) with respect to a given threshold ($RLU_{Ag^{**}}$) was obtained using the formula:

$$RLUAg^{**} = \frac{(RLUAg - RLUblank)}{Antigen\ threshold}$$

The result for a given antigen was considered positive if the RLU normalized value of a given antigen exceeded the positivity threshold established for it by the manufacturer. As described in the OIE report (OIE, 2019), two separate positivity thresholds were established by the manufacturer for each antigen allowing to obtain: (1) high specificity (HSp) data, and (2) high sensitivity (HSe) data. Data were analyzed according to the manufacturer's instruction (i.e., a serum is considered "positive" when at least two antigens are positive).

2.5. Antigen profiles analysis generated in Enferplex Bovine TB antibody test

In the Enfer11Ag TB kit, a serum sample is deemed positive when it gives a positive reaction to two or more antigens, whereas a serum sample that recognizes 0 or 1 antigen is deemed negative. As consequences, two to eleven antigens could be recognized by sera deemed positive in Enfer11Ag TB test and generate a "profiles of recognized antigens." These profiles are presented as a binary code for antigens 1 to 11 (0 for a negative antigen and 1 for a positive antigen) in this work. Only data obtained according to the HSp interpretation setting of the Enfer11Ag TB kit were used; however, no major differences were reported with the HSe setting. To assess if these profiles could be useful to distinguish false-positive sera versus true-positive sera (i.e., sera from non-infected and infected cattle showing at least 2 antigens positive in the Enfer11Ag TB test), profiles of recognized antigens from 89 Enfer11Ag TB-positive sera (SICCT positive and/or bacteriology positive) were analyzed and compared with the profiles of 9 false-positive sera identified through the non-infected herds of cattle tested (N = 308). To assess the diversity of the immune response to the 11 antigens

through bTB-outbreaks under study, profiles of all animals showing a positive result with the Enfer11Ag TB test in the positive herds (100/333) (independent of the skin test or bacteriological status) were then evaluated.

2.6. Evaluation of diagnostic tests and statistical analysis

The diagnostic specificity (DSp) values of the diagnostic techniques were calculated in using 308 sera from bTB free-cattle. Relative sensitivity (RSe) values were estimated in relation to: (1) 172 SICCT-positive animals originating from confirmed bTB outbreaks, and (2) the 38 bacteriology-positive animals. Wilson's 95% confidence intervals ($N < 40$) and Wald's 95% confidence interval ($N > 100$) were calculated. The distribution of S/P values in ELISA IDEXX was estimated using the Epanechnikov Kernel density module from package scikit-learn in Python. The kernel bandwidth was fixed to 0.026. Agreement values were calculated using the 333 sera collected from animals originating from bTB-outbreaks and the 308 sera from cattle originating from bTB-free herds (i.e., a total of 641 sera) and the kappa (k) statistic. A kappa of 1 indicates perfect agreement, while a kappa of 0 indicates no agreement. A 2×2 contingency table based on the Chi-square test (1 degree of freedom) was used to determine whether there was any difference between results obtained with the ELISA IDEXX and the Enfer11Ag TB tests and that of the manufacturers. The GraphPadPrism statistical tool was used (https://www.graphpad.com). For all statistical analyses, p-values (p) <0.05 were considered statistically significant. The seroprevalence of bTB estimated with the ELISA IDEXX and Enfer11Ag TB kits was evaluated for animals originating from three Belgian bTB-outbreaks. One of the four bTB-outbreaks (outbreak 2) included only animals ($N = 38$) selected because they were SICCT-positive, and was thus excluded from this analysis to avoid a bias calculating seroprevalence. Specificity and relative sensitivity values based on the serial interpretation of results from serological tests were calculated using the formulas: $Sp = 1\text{-}(1\text{-}Sp_a)x(1\text{-}Sp_b)$ and $Se = Se_a \ x \ Se_b$, where Sp_a, Se_a and Sp_b, Se_b are the specificity and relative sensitivity values estimated for the ELISA IDEXX and Enfer11Ag TB tests, respectively.

3. Results

3.1. Diagnostic performance of ELISA IDEXX

The diagnostic specificity and sensitivity values obtained for the ELISA IDEXX test are presented in Table 1, and are compared with the data provided by the manufacturer, IDEXX. The DSp value (97.1%) of the ELISA IDEXX was not significantly different ($p = 0.292$) to that of IDEXX based on samples from bTB-free areas. The obtained diagnostic sensitivity value relative to SICCT (36.6%) was significantly lower ($p < 0.0001$) than the RSe value (versus SICCT; 69.5%) given by IDEXX. However, the sensitivity value estimated relative to bacteriology data was no significantly different ($p = 0.425$) to that of the manufacturer IDEXX. When using all the data obtained from animals originating from bTB-outbreaks ($N = 333$) and bTB-free herds ($N = 308$), the distribution of the S/P values obtained with the ELISA IDEXX indicated that the cut-off value recommended by the manufacturer (≥0.30) appeared to be well-adapted for the cattle population in Belgium (Fig. 1).

3.2. Diagnostic performance of Enfer11Ag TB

The performance of the Enfer11Ag TB kit is presented in Table 2, and is compared with the data given by the manufacturer, EnferGroup. The DSp and RSe (versus SICCT and bacteriology) obtained with the Enfer11Ag TB test were significantly lower (DSp and RSe: $p < 0.001$) compared to those indicated by the manufacturer (OIE, 2019). The agreement between the HSp and HSe settings in the Enfer11Ag TB kit was excellent (k = 0.90; $p < 0.05$), showing that neither interpretation setting radically modified the obtained results. The sensitivity values relative to SICCT obtained with the Enfer11Ag TB kit were higher compared to those obtained with the ELISA IDEXX kit (Enfer11Ag TB HSp versus ELISA IDEXX: $p = 0.005$; Enfer11Ag TB HSe versus ELISA IDEXX: $p < 0.0001$). However, no significant difference was obtained for the sensitivity values estimated using the bacteriology as reference test (Enfer11Ag TB HSp versus ELISA IDEXX: $p = 0.23$; Enfer11Ag TB HSe versus ELISA IDEXX: $p = 0.09$). The DSp value obtained for the Enfer11Ag TB test under the HSp setting was identical to that obtained for the ELISA IDEXX (97.1%). However, the DSp value calculated for Enfer11Ag TB test with the HSe setting (95.1%) was lower, although this difference was not significant ($p = 0.2115$).

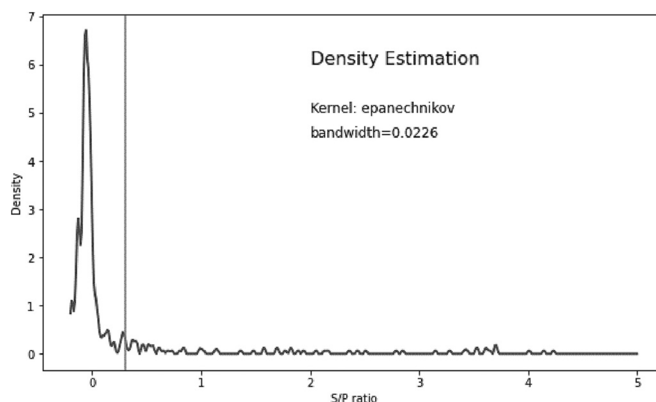

Fig. 1. Distribution of S/P values obtained with the ELISA IDEXX test when using all 641 sera from negative and positive herds of cattle studied in Belgium. Density curve represents a kernel estimation of the density. The grey vertical line shows the cut-off value recommended by the manufacturer (cut-off 0.30).

Table 1
Diagnostic specificity (DSp) and relative diagnostic sensitivity (RSe) values for the ELISA IDEXX test under conditions in Belgium, and data given by the IDEXX company. Chi-square test was performed to evaluate statistical differences of DSp and RSe data obtained in this study and those provided by the company. P-values <0.05 were considered as statistically different.

	DSp	RSe (versus SICCT)	RSe (versus bacteriology)
Current study data	$N = 308$ 97.1% (95% CI: 94.5-98.7)	$N = 172$ 36.6% (95% CI: 29.4-43.8)	$N = 38$ 57.9% (95% CI: 42.2-72.1)
Manufacturer data	$N = 1473$ 98.0% (95% CI: 97.5-98.4)	$N = 344$ 69.5% (95% CI: 64.4-74.1)	$N = 307$ 64.6% (95% CI: 59.7-69.5)
p-value (Chi2 test)	0.292	<0.0001	0.425

SICCT, Single Intradermal Comparative Cervical Tuberculin; CI, confidence interval.

Table 2
Diagnostic specificity (DSp) and relative diagnostic sensitivity (RSe) values for the Enfer11Ag TB kit under conditions in Belgium and data given by the EnferGroup company. Chi-square test was performed to evaluate statistical differences of DSp and RSe data obtained in this study and those provided by the company. P-values <0.05 were considered as statistically different.

	High specificity setting			High sensitivity setting		
	DSp	RSe (versus SICCT)	RSe (versus bacteriology)	DSp	RSe (versus SICCT)	RSe (versus bacteriology)
Current study data	N = 308 97.1% (95% CI: 94.5-98.7)	N = 172 51.7% (95% CI: 44.3-59.2)	N = 38 71.1% (95% CI: 55.2-83.0)	N = 308 95.1% (95% CI: 92.1-97.3)	N = 172 58.7% (95% CI: 51.4-66.1)	N = 38 76.3% (95% CI: 60.8-87.0)
Manufacturer data	N = 4258 99.7% (95% CI: 99.5-99.8)	*N* = 2076 89.6% (95% CI: 88.2-90.9)	*N* = 208 94.2% (95% CI: 91.1-97.4)	N = 4258 98.4% (95% CI: 97.8-98.0)	N = 2076 92.6% (95% CI: 91.5-93.8)	N = 208 94.2% (95% CI: 91.1-97.4)
p-value (Chi2 test)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0003

SICCT, Single Intradermal Comparative Cervical Tuberculin; CI, confidence interval.

Table 3
Profiles of antigens recognized by antibodies in the sera of non-infected cattle and infected-cattle that showed a positive response at 2 (or more) antigens, based on the high specificity interpretation setting of the Enfer11Ag TB kit. Underlined profiles were only found in "false-positive" sera.

	"False positive" sera	"True-positive" sera
[a] Antigen profile	[b] Non-infected cattle (n = 9)	[c] Infected-cattle (n = 89)
00000011000	1/9 (11.1%)	0/89 (0.0%)
00000100001	2/9 (22.2%)	0/89 (0.0%)
00010000010	1/9 (11.1%)	2/89 (2.2%)
00101000000	0/9 (0.0%)	1/89 (1.1%)
00110000000	0/9 (0.0%)	1/89 (1.1%)
00111000000	0/9 (0.0%)	1/89 (1.1%)
01111011000	0/9 (0.0%)	1/89 (1.1%)
10000000010	1/9 (11.1%)	0/89 (0.0%)
10000000100	0/9 (0.0%)	1/89 (1.1%)
10000100001	1/9 (11.1%)	0/89 (0.0%)
10010000000	2/9 (22.2%)	32/89 (36.0%)
10011000000	0/9 (0.0%)	3/89 (3.4%)
10110100000	0/9 (0.0%)	1/89 (1.1%)
10111000000	0/9 (0.0%)	4/89 (4.5%)
11010000000	1/9 (11.1%)	8/89 (9.0%)
11010001000	0/9 (0.0%)	1/89 (1.1%)
11010010000	0/9 (0.0%)	2/89 (2.2%)
11010011010	0/9 (0.0%)	1/89 (1.1%)
11010100001	0/9 (0.0%)	1/89 (1.1%)
11011000000	0/9 (0.0%)	7/89 (7.9%)
11011011000	0/9 (0.0%)	1/89 (1.1%)
11011110011	0/9 (0.0%)	1/89 (1.1%)
11110001000	0/9 (0.0%)	1/89 (1.1%)
11111000000	0/9 (0.0%)	10/89 (11.2%)
11111001000	0/9 (0.0%)	2/89 (2.2%)
11111010000	0/9 (0.0%)	2/89 (2.2%)
11111011000	0/9 (0.0%)	2/89 (2.2%)
11111011010	0/9 (0.0%)	1/89 (1.1%)
11111100000	0/9 (0.0%)	1/89 (1.1%)
11111111100	0/9 (0.0%)	1/89 (1.1%)

[a] Antigen (Ag) profiles are represented by a binary code (positive =1 negative =0) for Ag 1 to Ag 11 (i.e., position 1 to 11 in the profile).
[b] Number of sera among the negative population showing this profile.
[c] Number of sera among the positive (SICCT, Single Intradermal Comparative Cervical Tuberculin, and/or bacteriology) population showing this profile.

3.3. *Antigen profiles generated in Enferplex as a complementary diagnostic tool*

At first, using results previously generated by the evaluation of the specificity and sensitivity, profiles of recognized antigens of false-positive sera were compared to profiles of true-positive sera (i.e., sera from non-infected and infected cattle showing at least 2 antigens positive in the Enfer11Ag TB assay) to assess if these profiles might be used as complementary diagnostic tool. Four profiles (representing five out of nine sera) detected in the "false-positive" sera population were not present in the "true-positive" sera population (Table 3, underlined profiles). These profiles were mainly characterized by the positive response of antigens 1, 6, 10, and 11 with different associations.

Then, to assess the diversity of immune responses against the 11 antigens in population of infected cattle, profiles of positive sera in Enfer11Ag TB detected in the four bTB-outbreaks under study were analyzed. The results obtained for each bTB-outbreak are shown in Table 4. The profile presenting only the recognized antigens 1 and 4 was the most common in all outbreaks, except in outbreak 3 where it was not detected. The second most represented profile (except in outbreak 1) was the profile containing the first five antigens. Overall, the association of antigens 1 and 4 and the association the antigens from 1 to 5 were over-represented in the four studied bTB-outbreaks. Almost all the other profiles generated with the Enfer11Ag TB test included at least two of the first five antigens, in different combinations. Antigens from 6 to 11 were rarely detected, without the presence of antigens 1 or 4. Only one SICCT-negative and bacteriology-negative animal had a profile with just antigens 6 and 11.

Analysis of the profiles of 11 sera from SICCT-negative cattle belonging to positive herds indicated that five of them showed a positive response to the Enfer11Ag TB test involving at least four of the first five antigens, with previously identified profiles only in true-positive population of sera.

The existence of specific profiles for bacteriologically positive animals could not be assessed, because bacteriology positive animals that were not SICCT-positive ($N = 4$) were all negative in the Enfer11Ag TB test.

3.4. *Agreement between the two serological assays*

A moderate agreement was recorded for the two tests, with kappa values of 0.60 (ELISA IDEXX versus Enfer11Ag TB HSp) and 0.54 (ELISA IDEXX versus Enfer11Ag TB HSe) (Table 5). The profiles of recognized antigens by the antibodies of sera presenting a discordant result (65/641) between the two tests were analyzed. Out of the 17 sera giving a positive result with the ELISA IDEXX only, 52.9% (9/17) did not respond to any antigen in the Enfer11Ag TB kit, and 47.1% (8/17) only responded with one antigen (17.6% [3/17] antigen 1, 17.6% [3/17] antigen 3, and 11.8% [2/17] antigen 4). In contrast, out of the 48 sera responding negatively in the ELISA IDEXX, but positively in the Enfer11Ag TB kit, almost half (22/48; 45.8%) showed a positive result with both antigens 1 and 4. The others showed various profiles of positive antigens. Of note, out of the 4 sera from animals that were only bacteriology positive, none were positive in Enfer11Ag TB (only one serum was positive for the antigen 4), which contrasted to ELISA IDEXX (1/4).

Table 4
Profiles of recognized antigens by antibodies in the sera for all cattle originating from four bTB-outbreaks in Belgium that were deemed positive with the Enfer11Ag TB kit according to the high specificity interpretation setting (100/333). Profiles obtained in each bTB-outbreak are also shown.

[a]Antigen profile	Outbreak 1 ($n = 10$)	Outbreak 2 ($n = 21$)	Outbreak 3 ($n = 13$)	Outbreak 4 ($n = 56$)	Total of 4 bTB-outbreaks ($n = 100$)
10010000000	40.0% (4/10)	28.6% (6/21)	0.0% (0/13)	42.9% (24/56)	34.0% (34/100)
11011000000	10.0% (1/10)	14.3% (3/21)	7.7% (1/13)	5.4% (3/56)	8.0% (8/100)
10011000000	10.0% (1/10)	4.3% (1/21)	0.0% (0/13)	1.8% (1/56)	3.0% (3/100)
11111010000	10.0% (1/10)	0.0% (0/21)	0.0% (0/13)	1.8% (1/56)	2.0% (2/100)
11111000000	0.0% (0/10)	14.3% (3/21)	46.2% (6/13)	7.1% (4/56)	13.0% (13/100)
00101000000	10.0% (1/10)	0.0% (0/21)	0.0% (0/13)	0.0% (0/56)	1.0% (1/100)
00000100001	10.0% (1/10)	0.0% (0/21)	0.0% (0/13)	0.0% (0/56)	1.0% (1/100)
11010000000	10.0% (1/10)	0.0% (0/21)	0.0% (0/13)	14.3% (8/56)	9.0% (9/100)
11111001000	0.0% (0/10)	9.5% (2/21)	0.0% (0/13)	0.0% (0/56)	2.0% (2/100)
00010000010	0.0% (0/10)	4.3% (1/21)	7.7% (1/13)	0.0% (0/56)	2.0% (2/100)
11111111100	0.0% (0/10)	4.3% (1/21)	0.0% (0/13)	0.0% (0/56)	1.0% (1/100)
10111000000	0.0% (0/10)	4.3% (1/21)	0.0% (0/13)	5.4% (3/56)	4.0% (4/100)
10000000100	0.0% (0/10)	4.3% (1/21)	0.0% (0/13)	0.0% (0/56)	1.0% (1/100)
11010011010	0.0% (0/10)	4.3% (1/21)	0.0% (0/13)	0.0% (0/56)	1.0% (1/100)
11111011000	0.0% (0/10)	0.0% (0/21)	7.7% (1/13)	1.8% (1/56)	2.0% (2/100)
01111011000	0.0% (0/10)	0.0% (0/21)	7.7% (1/13)	0.0% (0/56)	1.0% (1/100)
11010100001	0.0% (0/10)	0.0% (0/21)	7.7% (1/13)	0.0% (0/56)	1.0% (1/100)
00010100001	0.0% (0/10)	0.0% (0/21)	7.7% (1/13)	0.0% (0/56)	1.0% (1/100)
10000000010	0.0% (0/10)	0.0% (0/21)	7.7% (1/13)	0.0% (0/56)	1.0% (1/100)
11111011010	0.0% (0/10)	0.0% (0/21)	0.0% (0/13)	1.8% (1/56)	1.0% (1/100)
11010010000	0.0% (0/10)	0.0% (0/21)	0.0% (0/13)	3.6% (2/56)	2.0% (2/100)
11010001000	0.0% (0/10)	0.0% (0/21)	0.0% (0/13)	1.8% (1/56)	1.0% (1/100)
00111000000	0.0% (0/10)	0.0% (0/21)	0.0% (0/13)	1.8% (1/56)	1.0% (1/100)
11110001000	0.0% (0/10)	0.0% (0/21)	0.0% (0/13)	1.8% (1/56)	1.0% (1/100)
10110100000	0.0% (0/10)	0.0% (0/21)	0.0% (0/13)	1.8% (1/56)	1.0% (1/100)
00110000000	0.0% (0/10)	0.0% (0/21)	0.0% (0/13)	1.8% (1/56)	1.0% (1/100)
11111100000	0.0% (0/10)	0.0% (0/21)	0.0% (0/13)	3.6% (2/56)	2.0% (2/100)
11011110011	0.0% (0/10)	0.0% (0/21)	0.0% (0/13)	1.8% (1/56)	1.0% (1/100)

[a] Profiles of antigens (Ag) are represented by a binary code (positive =1 negative =0) for Ag 1 to Ag 11 (i.e. position 1 to 11 in the profile).

Table 5
Agreement between the ELISA IDEXX test and Enfer11Ag TB test based on: (A) high specificity interpretation, and (B) high sensitivity interpretation settings.

A

		Enfer11Ag TB High specificity setting		
		POSITIVE	NEGATIVE	Total
ELISA IDEXX	POSITIVE	61	17	78
	NEGATIVE	48	515	563
	Total	109	532	641

B

		Enfer11Ag TB High sensitivity setting		
		POSITIVE	NEGATIVE	Total
ELISA IDEXX	POSITIVE	63	15	78
	NEGATIVE	65	498	563
	Total	128	513	641

Moderate agreement with a Kappa coefficient value of 0.60 (95% CI: 0.52–0.67); $p < 0.0001$.
Moderate agreement with a Kappa value of 0.54 (95% CI: 0.47–0.62); $p < 0.0001$.

3.5. *Seroprevalence of the bovine tuberculosis estimated using the two kits*

The seroprevalence of bovine tuberculosis estimated with the ELISA IDEXX and Enfer11Ag TB kits was evaluated for animals originating from three Belgian bTB-outbreaks ($N = 295$). The seroprevalence calculated with the ELISA IDEXX test was 6.3–29.4%, whereas it was 9.1-47.1% and 9.1–54.6% for the HSp and HSe settings, respectively, of the Enfer11Ag TB test (Table 6).

4. Discussion

Published data and tests used by the surveillance programs to diagnose bTB are currently not able to identify it in all animals at all stages of infection. Thus, a diagnostic approach integrating multiple diagnostic methods could enhance the efficiency and reliability of detection in infected animals. This study evaluated the performance of two commercial serological diagnostic assays, approved by the World Organization of Animal Health (WOAH), in a Belgian bovine population.

The DSp value obtained with the ELISA IDEXX kit in the current study was similar to that indicated by IDEXX and some previously reported values (OIE, 2012; Waters et al., 2011), but was lower compared to other studies (Casal et al., 2014; Hirpa et al., 2014). Waters and colleagues showed that the specificity of the ELISA IDEXX kit varies across bTB-free populations of cattle depending on geographic area and, possibly, local microbiome (Waters et al., 2011). In contrast, the DSp values obtained in the current study with the Enfer11Ag TB kit (HSp and HSe settings) were lower compared to the high values of specificity reported by the EnferGroup manufacturer (OIE, 2019). To date, no study has been published evaluating the Enferplex TB kit (using 11 antigens); however previous studies using modified versions (from 4 to 25 antigens) of the current kit showed that specificity values range from 79.6% to 100% (Casal et al., 2014; McCallan et al., 2021; Whelan et al., 2008, 2010). Differences in the number of antigens used, interpretation settings and populations tested might explain these variability and discrepancy. Moreover, not significant differences were reported in data published by EnferGroup company (OIE, 2019) between diagnostic specificity values estimated from boosted and non-boosted bTB-free animals ($N = 4258$ bTB-free animals non-boosted: Sp = 98.4% (HSp), Sp = 99.7% (HSe) versus $N = 161$ bTB-free animals boosted: Sp = 100.0% with HSp and HSe settings; $p > 0.05$). That suggests blood sampling from bTB-free cattle within or out the amnestic window should not influence specificity values of this study. While DSp values calculated for ELISA IDEXX and the HSp setting of the Enfer11Ag TB were identical, it was

Table 6
Seroprevalence of bTB estimated using ELISA IDEXX and Enfer11Ag TB tests from bovine sera originating from bTB-outbreaks in Belgium.

	[a]Total of animals/herd	[b]ELISA IDEXX Seroprevalence (%)	[c]Enfer11Ag TB HSP Seroprevalence (%)	[d]Enfer11Ag TB HSE Seroprevalence (%)
Outbreak 1	34	14.7% (5/34)	29.4% (10/34)	32.3% (11/34)
Outbreak 3	142	6.3% (9/142)	9.1% (13/142)	9.1% (13/142)
Outbreak 4	119	29.4% (35/119)	47.1% (56/119)	54.6% (65/119)
TOTAL	**295**	**16.6% (49/295)**	**26.8% (79/295)**	**30.2% (89/295)**

bTB, bovine tuberculosis; HSP, high specificity; HSE, high sensitivity.
[a] Total number of sera collected per herd.
[b] Total Number of positive animals in the herd with the ELISA IDEXX test.
[c] Total Number of positive animals in the herd with the Enfer11Ag TB test using the HSp setting.
[d] Total Number of positive animals in the herd with the Enfer11Ag TB test using the HSe setting.

not the case for the HSe setting of the Enfer11Ag TB that showed a lower DSp value compared to ELISA IDEXX. This difference was probably due to the "HSe setting," in which the specificity of some antigens was sacrificed by reducing their individual positivity threshold to improve their sensitivity.

The false-positive reactions observed in the present study reduced the DSp of serological tests, and might be associated to exposure to non-tuberculous mycobacteria (NTM). For instance, infections with high doses of *M. kansasii* may generate false positive results, based on MPB83 and MPB70 proteins being conserved (>81% and 77% of identity sequence, respectively) in *M. kansasii* (Waters et al., 2011). Other mycobacterial species have been isolated in cattle throughout Europe, including *M. avium* subsp. *Avium*, *M. avium* subsp. *hominissuis* and *M. nonchromogenicum* (Biet and Boschiroli, 2014; Varela-Castro et al., 2022). These species might react with the antigens used in the serological tests, as several immunogenic antigens of *M. bovis* are shared with some of these NTM species (Gcebe et al., 2016; Infantes-Lorenzo et al., 2017). As consequences, and to increase the specificity of the Enfer11Ag TB kit, we could consider to modify individual positivity thresholds of antigens of the kit to adapt it more at the Belgian bovine population.

The sensitivity values relative to SICCT-positive animals and bacteriology-positive animals obtained in this study were overall lower compared to those established by IDEXX and EnferGroup companies. These discrepancies might be due to population of cattle used along with the boost status from animals tested by companies. Several authors showed that the sensitivity of the ELISA IDEXX test for bTB detection in cattle is highly variable with values reported between 9% and 77% (Trost et al., 2016; Waters et al., 2011). For the Enferplex TB kit including 11 antigens, as mentioned previously, no study evaluating the sensitivity of this test is already published. To date, studies using the multiplexed method developed by EnferGroup company, with 6 to 25 antigens, showed generally higher sensitivity compared to results obtained in the current study (Casal et al., 2014; Whelan et al., 2010). Overall the difference in sensitivity observed with both tests (Enfer11Ag TB and ELISA IDEXX) might be explained by the stage of infection of animals tested. For instance, different stages of infection in herds of cattle might be induced by high variability in *M. bovis*-susceptibility and the development of the immune response in infected cattle due to multiple factors (for description see, Bermingham et al., 2014; Broughan et al., 2016; Dean et al., 2005; Humblet et al., 2009), along with the slow evolution of the disease (Cassidy, 2006; Domingo et al., 2014). However, in the case of the different versions of Enferplex tests compared, difference in the number of antigens used (6 to 25 antigens previously versus 11 antigens in this study) along with different cut-off might also explain the different sensitivity values obtained (Casal et al., 2014; Whelan et al., 2010).

Due to the highly variable kinetics of antibody responses in *M. bovis* infected animals, multiplexed tests might be considered advantageous for bTB diagnosis (Fifis et al., 1992; Lyashchenko et al., 1998). The current study showed that the Enfer11Ag TB test had significantly higher sensitivity compared to the ELISA IDEXX test, particularly for SICCT-positive animals. This difference was attenuated when using bacteriology-positive animals for both tests. These results might indicate that there is higher diversity among the stages of infection in SICCT-positive animals, which would be more likely to be detected when using multiple antigens and with different conformations (e.g., peptides, recombinant proteins, and fusion proteins). The more the disease progresses, the greater the probability of detecting sero-dominant proteins (e.g., MPB70 and/or MPB83), which are common in both tests (Lightbody et al., 1998; Lyashchenko et al., 2004), and this can reduce the difference observed between sensitivity values.

ELISA IDEXX is a serological assay that detects the antibodies against MPB70 and MPB83 proteins in peptide form (Waters et al., 2011). In contrast, Enfer11Ag TB is a multiplexed immunoassay that simultaneously detects the antibodies against multiple antigens of *M. bovis*, including MPB70 and MPB83, as peptide and/or recombinant protein forms (Casal et al., 2014; Whelan et al., 2008). Although the two serological tests used the two sero-dominant antigens (MPB70 and MPB83), they only showed a moderate agreement. In contrast, two previous studies reported substantial agreement between the ELISA IDEXX test and different version of the Enferplex method using six and four antigens, respectively, mainly immuno-dominant antigens of *M. bovis* i.e. MPB70/83 and ESAT6/CFP10 proteins (Casal et al., 2014; McCallan et al., 2017). When we performed the agreement analysis when considering only first five antigens of the Enfer11Ag TB test, supposed to include sero-dominant antigens, the agreement between both tests became substantial ($k = 0.64$ with the HSp setting; $k = 0.61$ with the HSe setting). Thus, the moderate agreement obtained in our study was not due to the technological differences of the two tests (i.e. the optical density versus the luminescent measurement) but more to characteristics of the Enfer11Ag TB kit used in this study.

The analysis of profiles of recognized antigens showed that the recognition of infected animals in our study involved mainly the antigens from 1 to 5. Although the EnferGroup company has not publicly disclosed the nature of antigens used in the Enfer11Ag TB kit, antigens 1 to 5 likely include the sero-dominant antigens MPB 70 and 83 in different forms (recombinant protein and/or peptides), based on our results and existing studies (Casal et al., 2014; Whelan et al., 2008, 2010). Antigens 6 to 11 were minimally or not recognized in infected cattle. However, these antigens might have the potential to detect infected animals at stages of infection that were not captured in the current study. Unfortunately, we currently were not able to use the profiles of recognized antigens to add a diagnostic value to this multiplexed test through distinguishing false positive and true positive results, even though a few unique profiles were detected in the negative population. However, although their status infectious were not confirmed, five out eleven sera from SICCT-negative animals originating from bTB-outbreaks showed profiles associated to infected animals, suggesting that these cattle might be at an anergic stage. More data are required to confirm the obtained results.

In the present study, around half of SICCT-positive animals were

positive with the Enfer11Ag TB multiplexed serological assay, and around one third were positive with the ELISA IDEXX serological assay. This observation indicates that positive serological results do not necessarily match with absence results in the cellular diagnostic method and might expand the diagnostic value of serological assays to other animals than skin tested non-reactor cattle. Our estimates of seroprevalence for different bTB-outbreaks in Belgium also showed that, for some herds, intra-herd prevalence should be compatible with the serological detection of infected animals at the herd level when using the ELISA IDEXX and/or Enfer11Ag TB tests. Of note, sera from animals used in this study to determine the sensitivity of both serological assays were collected within a window of 30 days following the SICCT test. It is well accepted that the blood sampling carried out within anamnestic window improves serologic results (Casal et al., 2014). Accordingly, it should be expected that the relative sensitivity values of serological assays evaluated in this study would be decreased when using blood sampling from non-boosted cattle by a prior skin test (OIE, 2019; McCallan et al., 2021).

The assessment of performance of ELISA IDEXX and Enfer11Ag TB indicated that sensitivity of Enfer11Ag TB is higher than the one of ELISA IDEXX, with specificity values similar. Of course, the predictive value of these two assays is modified by the context of their usage (i.e., global screening versus risk-based survey), and if they are used with/without another diagnosis test, such as the IFN-gamma assay. However, in OTF-certified countries with a low bTB prevalence, such as Belgium (<0.1%), reducing the number of false-positive results is important, as it is a source of important economic loss. To increase overall diagnostic specificity, serial interpretation of results of the ELISA IDEXX and Enfer11Ag TB tests was performed. Although, serial interpretation of both assays decreased the overall relative sensitivity, specificity was raised to 99.9% when using the HSp setting of the Enfer11Ag TB kit, decreasing radically the false-positive rate what is essential in a context of low prevalence. Furthermore, it is likely that bTB-outbreaks with a high sero-prevalence (as bTB-outbreaks 1 and 4 in this study) might still be correctly identified by the serial interpretation of serological results. Serological detection might thus stay interesting as additional tools to detect herds with high bTB prevalence which constitute a source of maintenance and propagation of the disease.

Both serological assays, with the serial interpretation of results, have been implemented following the decision of Belgian policy makers in the new Belgian surveillance program initiated in 2021, in a context of risk-based survey. Additional analyzes are currently being carried out in order to assess the impact of the implementation of serological tests as complementary tests to the IFNɣ assay in the new Belgian surveillance program.

5. Conclusions

This study assessed the performance of the ELISA IDEXX kit and the Enferplex TB kit (including 11 antigens) in cattle herds in Belgium. We recorded high specificity values for both serological assays; however, the multiplexed Enfer11Ag TB test had higher relative sensitivity when tested in series with the SICCT test compared to the ELISA IDEXX test. The profiles of recognized antigens generated by the Enfer11Ag TB test for negative and positive cattle populations were interesting. However, at present, these profiles cannot be used as additional diagnostic tools to distinguish false and true positive sera, with more data being required. The strategic use of serially interpreting both serological tests considerably improved the global specificity of the bTB diagnosis, which is essential in a country where prevalence is low, such as Belgium.

Declaration of Competing Interest

All kits used in this study were purchased by Sciensano. EnferGroup and IDEXX companies played no role in the study design nor in the collection, analysis, and interpretation of data, nor in the decision to submit the manuscript for publication. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

The research that yielded these results, was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment through the contract RT 18/01 DIBOTUB. Preliminary results were presented as a poster at the Seventh International Conference on *Mycobacterium bovis*, Galway, 7-10 June 2022. We thank members from "Association Régionale de Santé et d'Identification Animales" (ARSIA) for providing the serum samples used in this study. We also thank Ana Soares, Philippe Vannoorenberghe, Damien Desqueper, and Willem Van Campe for providing technical assistance.

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