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journal homepage: www.elsevier.com/locate/vetimmField application of immunoassays for the detection of *Mycobacterium bovis* infection in the African buffalo (*Syncerus caffer*)E.M.D.L. van der Heijden^{a,b,*}, A.O. Jenkins^{a,b}, D.V. Cooper^c, V.P.M.G. Rutten^{a,b}, A.L. Michel^a^a Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, Pretoria, South Africa^b Division of Immunology, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL, Utrecht, The Netherlands^c Ezemvelo KwaZulu-Natal Wildlife, Private Bag 01, St. Lucia, 3936, South Africa

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ABSTRACT

The African buffalo (*Syncerus caffer*) is considered the most important maintenance host of bovine tuberculosis (BTB) in wildlife in Southern Africa. The diagnosis of *Mycobacterium bovis* infection in this species mostly relies on the single intradermal comparative tuberculin test (SICTT). As an alternative, the BOVIGAM[®] 1G, an interferon-gamma (IFN- γ) release assay, is frequently used. The test performance of cell-mediated immunity (CMI-) and humoral immunity (HI-) based assays for the detection of *M. bovis* infections in buffaloes was compared to identify the test or test combination that provided the highest sensitivity in the study. Buffaloes were sampled during the annual BTB SICTT testing in the Hluhluwe-iMfolozi-Park (KwaZulu-Natal, South Africa) during June 2013. A total of 35 animals were subjected to the SICTT, 13 of these tested positive and one showed an inconclusive reaction. CMI-based assays (BOVIGAM[®] 1G (B1G) and BOVIGAM[®] 2G (B2G)) as well as a serological assay (IDEXX TB ELISA) were used to further investigate and compare immune responsiveness. Thirteen SICTT positive buffaloes and one inconclusive reactor were slaughtered and a post-mortem (PM) examination was conducted to confirm BTB. Lesions characteristic of BTB were found in 8/14 animals (57.1%). Test results of individual assays were compared with serial and parallel test interpretation and the sensitivity was calculated as a percentage of test positives out of the number of SICTT positive animals with granulomatous lesions (relative sensitivity). The B1G assay showed the highest individual sensitivity (100%; 8/8) followed by the B2G assay (75%; 6/8) and the IDEXX TB ELISA (37.5%; 3/8). Therefore, using in parallel interpretation, any combination with the B1G showed a sensitivity of 100% (8/8), whereas combinations with the B2G showed a 75% sensitivity (6/8). Out of the 21 SICTT negative animals, 7 animals showed responsiveness in the B2G or IDEXX TB ELISA. In conclusion, this study has shown that the BOVIGAM[®] IFN- γ assay had the highest test performance.

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1. Introduction

Tuberculosis is a debilitating, chronic, infectious disease that may be caused by any of several closely related bacilli of the *Mycobacterium tuberculosis* complex (MTBC) (O'Reilly and Daborn, 1995) and remains a major global health issue today (World Health Organization, 2014). *Mycobacterium bovis* (*M. bovis*), the MTBC species with the broadest host range and a zoonotic agent is the main causative agent of bovine tuberculosis (BTB) in cattle (O'Reilly and Daborn, 1995). The African buffalo (*Syncerus caffer*) is considered the most important maintenance host of *M. bovis* in Southern Africa and as most mammals are susceptible, the list of spillover hosts is rather extensive and includes several valuable species of wildlife (de Lisle et al., 2002, Michel et al., 2006, Renwick

Abbreviations: B1G, BOVIGAM[®] 1G; B2G, BOVIGAM[®] 2G; BTB, bovine tuberculosis; CMI, cell-mediated immunity; HI, humoral immunity; HiP, Hluhluwe-iMfolozi-Park; IFN- γ , interferon-gamma; KNP, Kruger National Park; MTBC, *Mycobacterium tuberculosis* complex; NVL, No visible lesions; OD, optical density; PPD, purified protein derivative; PPD-A, purified protein derivative of *Mycobacterium avium*; PPD-B, purified protein derivative of *Mycobacterium bovis*; PPD-F, purified protein derivative of *Mycobacterium fortuitum*; PWM, pokeweed mitogen; RD, region of difference; SICTT, single intradermal comparative tuberculin test; S/P, sample/positive control; TB, tuberculosis.

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et al., 2007). The endemic occurrence of *M. bovis* infections in the Hluhluwe-iMfolozi-Park (HIP) and the Kruger National Park (KNP) in South Africa together with the fact that the prevalence of BTB has been rising has grave implications as it is likely to compromise (i) conservation efforts, especially so in vulnerable or endangered species (Espie et al., 2009), (ii) BTB control efforts in cattle due to spillback from buffaloes (Musoke et al., 2015), (iii) public health due to the risk of zoonotic tuberculosis (TB) (Muller et al., 2013) and (iv) (inter-) national trade due to stringent restrictions on trade in wildlife from BTB infected parks (Michel et al., 2006).

The diagnosis of *Mycobacterium bovis* infection in buffaloes mostly relies on the single intradermal comparative tuberculin test (SICTT) (Michel et al., 2011, DAFF, RSA, 2013). The SICTT, however, comes with several drawbacks as sensitivity and specificity are known to be highly variable (Monaghan et al., 1994, de la Rua-Domenech et al., 2006) and the animals need to be held in captivity for a minimum of 72 h and chemically manipulated twice (Grobler et al., 2002). The interferon-gamma (IFN- γ) release assay (BOVIGAM[®] 1G (B1G)) is increasingly used for diagnosis of *M. bovis* infections in cattle and has been optimized for application in African buffaloes by Michel et al. (2011). This improved protocol has offered the opportunity to achieve higher test specificity (93.9%) by the introduction of PPD-F (purified protein derivative of *M. fortuitum*) as a locally representative antigen preparation of non-tuberculous mycobacteria (NTM) (Michel et al., 2011). The BOVIGAM[®] 2G (B2G) assay was designed to increase specificity by inclusion of peptide cocktails comprised of antigens encoded in the RD1 (region of difference) gene region considered to be unique for MTBC species; PC-EC contains ESAT-6- and CFP-10-derived peptides (Life Technologies, 2015a) and PC-HP additionally contains 4 mycobacterial genes (including Rv3615 (Goosen et al., 2014)) (Life Technologies, 2015b). In order to effectively monitor and control BTB it is of utmost importance that diagnostic assays are available which are accurate and fit for the test purpose. Furthermore, these assays should allow for adaptive test interpretation when different epidemiological settings require either optimum sensitivity or specificity. In the HIP, the purpose of the BTB monitoring program is to reduce overall and herd prevalence of BTB in the park (Michel et al., 2006), requiring highly sensitive diagnostics.

The objective of the current study was to assess the array of diagnostic options currently available commercially for the detection of *M. bovis* infection in buffaloes, in order to identify the test or test combination that could achieve the highest sensitivity, relative to the SICTT.

2. Materials and methods

2.1. Location

The Hluhluwe-iMfolozi Park is situated in the KwaZulu-Natal province of South Africa and covers approximately 96,000 ha (Ezemvelo KZN Wildlife, 2015). There are approximately 5,600 free-roaming African buffaloes in the park. The buffalo holding bomas were set up at the Nselweni site next to the permanent bomas in the iMfolozi section of the park (28° 18' 05.7" S 31° 53' 29.3" E).

2.2. Capture and immobilisation procedure

Animals were mass captured using a plastic boma set-up (Kock and Burroughs, 2012) on day 0. Prior to handling, all animals were immobilized using a mixture of the opioid derivative etorphine hydrochloride (M99, Novartis Animal Health, Isando, South Africa) and the butyrophenone tranquilizer azaperone (Stresnil, Janssen Pharmaceutica, Woodmead, South Africa). Animals were then given ear tags and brands (X; V) for the purpose of identification.

After application of the skin test and sampling, the animals were reversed using diprenorphine hydrochloride (M5050, Novartis Animal Health, Isando, South Africa). The same procedure was used for immobilisation and reversal of the animals during reading of the skin test.

2.3. SICTT

The SICTT was performed according to the OIE standards (World Organisation for Animal Health, 2014). Briefly, an area of skin (3 × 5 cm) was shaved on both sides of the neck, skin fold thickness at both injection sites was measured with callipers and 3000 IU of PPD-B (purified protein derivative of *M. bovis*) (Prionics AG, Lelystad, the Netherlands) and 2500 IU of PPD-A (purified protein derivative of *M. avium*) (Prionics AG, Lelystad, the Netherlands) were injected intradermally in the left and right side of the neck, respectively. Thirty-five animals were skin tested and injected on day 1. Reading of the SICTT took place 72 h (day 4) after injection of the tuberculin PPDs (purified protein derivatives). Interpretation of the SICTT was done according to the guidelines for interpretation of infected herds as validated locally by the State Veterinary Office of Hluhluwe (McCall, unpublished data). Briefly, an animal is considered positive when the skin thickness increase at the bovine injection site is >4 mm and the difference between the skin thickness at bovine and avian sites is ≥ 2 mm; the reaction is considered inconclusive when the bovine site shows an increase of >4 mm and the difference between the bovine and avian sites is between one and two millimetres; the reaction is considered negative if the difference between the bovine and avian sites is ≤ 0 -1 mm, even if the bovine site shows an increase of >4 mm.

2.4. Sample collection

Whole blood was collected from the jugular vein using a vacuum system. On day 1, prior to tuberculin injection, whole blood was collected into serum tubes for serological assays. Serum samples were left to clot at ambient temperature for 24 h and sera were harvested and frozen at -20°C until further analysis. On day 4, whole blood was collected into heparin tubes for IFN- γ assays. Heparin samples were processed within 4–6 h after collection.

2.5. Bovine IFN- γ release assays

The stimulations for the B1G assay were carried out in 24-well cell culture plates (Cellstar[®] Greiner Bio One). Undiluted heparinised blood was aliquoted into 1.5 ml per well and stimulated with 50 μl pokeweed mitogen (PWM) (5 $\mu\text{g}/\text{ml}$) (internal positive control), 60 μl PPD-A (1000 IU/ml), 30 μl PPD-B (600 IU/ml) (Prionics AG, Lelystad, the Netherlands) and 25 μl PPD-F (0.5 mg/ml) (ARC-Onderstepoort Veterinary Institute). An unstimulated aliquot of whole blood served as an internal negative control; according to Michel et al. (2011). The samples were incubated at 37°C for 24 h. The supernatants were harvested and stored at -20°C until further analysis. The detection of IFN- γ and the interpretation of results were conducted according to the manufacturer's protocol (Prionics AG, Schlieren-Zurich, Switzerland). Samples were considered valid for analysis if OD-PWM was ≥ 0.45 and if OD-neg control was ≤ 0.35 .

The stimulations for the B2G assay were carried out in triplicate in 96-well cell culture plates (Cellstar[®] Greiner Bio One). Undiluted heparinised blood was aliquoted into 250 μl per well and stimulated with the peptide cocktails PC-EC (0.1 mg/ml) and PC-HP (0.1 mg/ml) (Prionics AG, Schlieren-Zurich, Switzerland) as well as PPD-A (1000 IU/ml), PPD-B (600 IU/ml) (Prionics AG, Schlieren-Zurich, Switzerland) and PWM (5 $\mu\text{g}/\text{ml}$) (internal positive control).

in volumes of 25 µl per antigen. An unstimulated aliquot of whole blood served as an internal negative control. The samples were incubated at 37 °C for 24 h. The supernatants were harvested and stored at –20 °C until further analysis. The detection of IFN-γ and the interpretation of results were conducted according to the manufacturer's protocol (Prionics AG, Schlieren-Zurich, Switzerland). Criteria for valid samples controls were as described for the B1G assay.

2.6. Serological assay

In the IDEXX TB ELISA, sera were tested in three dilutions; undiluted, 1:2 dilution and 1:10 dilution, using the sample diluent of the test kit. For the remainder of the assay the protocol was followed as supplied by the manufacturer. Criteria for the test validity were an OD-value of ≥ 0.3 for the positive control and ≤ 0.2 for the negative control. The interpretation was done according to the manufacturer's protocol, using S/P (sample/positive control) ratios.

2.7. Post-mortem examination

All animals testing positive or inconclusive on the SICTT were culled and a post-mortem (PM) examination was carried out. During PM examination the carcasses were examined for lesions characteristic of BTB as described previously by de Klerk et al., 2010. Lymph nodes of the head and lungs were excised from the carcasses and subsequently sliced into 1–2 mm sections using a scalpel blade for thorough inspection and palpation (de Klerk et al., 2010). The lungs were removed from the carcasses and inspected and palpated as a whole as well as after incision in all lobes (de Klerk et al., 2010). The scoring of macroscopic lesions was slightly adapted (Table 1) from de Klerk et al., 2010.

2.8. Data analysis

As only SICTT positive animals were culled, further analysis of test performances was relative to that of the SICTT. Animals presenting with tuberculous lesions at post-mortem examination were considered true BTB positives and only these were used for further analysis of relative test sensitivity. Relative test sensitivity was calculated as the proportion of true positives correctly classified by the test. Relative sensitivity for the B2G assay was calculated for three test formats, considering that it can be performed using either the PPD antigens or the peptide cocktails in isolation as stimulatory antigens, or they can all be applied in parallel. The assay results of all tests for combined application were interpreted either in parallel or in series to determine whether these selected combinations could improve overall sensitivity (Lewis et al., 2008). Test agreement was determined by Cohen's kappa coefficient or κ , which allows comparison of two different methods of measurement (Petrie and Watson, 2006).

Table 1

Scoring of macroscopic lesions for BTB. Lymph nodes and lung tissue were carefully examined and scored according to the scoring system adapted from de Klerk et al. (2010).

Score	Description
0	No visible lesions
0.5	Reactive lymph node but no visible lesions found
1	One to five lesions of 1–2 mm
2	One to five lesions of >2 mm or >5 lesions of 1–2 mm
3	TB-like lesions in the majority of tissue slices but some healthy tissue observed
4	Majority of sectioned tissue presented with TB-like lesions

3. Results

3.1. SICTT

Thirteen out of thirty-five animals were test-positive in the SICTT (Table 2a), using the criteria set out. One animal's result was inconclusive. The remaining twenty-one animals tested negative with differences in the increase in skin thickness between bovine and avian of ≤ 0.1 mm. (Data not shown.) Positive responder frequency of the SICTT was 100% (Table 3) as culling was biased towards SICTT positive animals and thus no PM data for SICTT negative animals could be collected.

3.2. Post-mortem examination

Post-mortem examination was carried out on the 14 animals that tested positive or inconclusive on the SICTT. Lesions characteristic of BTB were found in 8 out of these 14 animals (57.1%). The animal that had an inconclusive test result on the SICTT did not have lesions. Lesions were mainly found in the bronchial and mediastinal lymph nodes, but also lung tissue was frequently affected (Fig. 1). Most of the affected carcasses had lesion scores of 1–2, one carcass presented with a lesion score of 4 (Table 2a).

3.3. IFN-γ assays

Twelve out of thirty-five animals (34.3%) tested positive in the B1G (Table 2a), resulting in a relative sensitivity of the B1G assay of 100% (8/8) (Table 3). Four animals tested positive in the B1G but did not show lesions on PM.

Thirteen out of thirty-five animals (37.1%) tested positive in the B2G (Table 2a). The classic antigen preparations PPD-A and PPD-B detected *M. bovis* reactivity in 13 animals. There was reactivity to the peptide cocktail PC-EC in 4 animals, whereas 5 animals showed reactivity to the peptide cocktail PC-HP. Relative sensitivity of the B2G assay, taking reactivity to both the PPDs and the peptide cocktails into account, was 75% (6/8) (Table 3). Whereas the relative sensitivity when taking into account the PPDs alone was also 75% (6/8), for both cocktails it was 50% (4/8) and when taking into account either PC-EC or PC-HP reactivity the sensitivities were 37.5% (3/8) and 50% (4/8), respectively (Table 3). Three animals tested positive in the B2G using the PPDs, but were negative on all other assays including SICTT (no PM data available) (Table 2b).

3.4. Serology

The S/P ratios for all diluted samples (both 1:2 as well as 1:10) yielded negative test outcomes in the IDEXX TB ELISA. The undiluted samples did yield S/P ratios above the cut-off levels in 9 out of thirty-five animals (25.7%) with S/P-levels ranging between 0.31 and 1.31. The relative sensitivity of the IDEXX TB ELISA was found to be 37.5% (3/8) (Table 3). Four animals tested positive in the IDEXX TB ELISA, but were negative in all other assays including SICTT (no PM data available) (Table 2b).

3.5. Single application of tests

Overall, the SICTT and the B1G assay showed higher individual sensitivity than the other assays, both with a relative sensitivity of 100% (Table 3). Fourteen SICTT negative animals tested negative for all other assays, whereas 7 animals reacted in either the B2G or the IDEXX TB ELISA (Table 2b). The B2G assay is less sensitive than the SICTT and the B1G assay, with a relative sensitivity of 75%. The B2G assay was found to be more sensitive when using PPD-A and PPD-B (75%) than when based on the peptide cocktails, which did not add to the overall sensitivity of the B2G assay (Table 3).

Table 2a

Results of all assays for all culled animals. All results in bold reflect positive results or values above cut-off levels. C = circumscribed; D = diffuse; N = necrosis; O = oedematous, ∞ = immeasurable.

Ear tag #	SICTT		Remarks	B1G (ODbov- ODav)	B2G A&B (ODbov- ODav)	B2G HP (ODhp- nil)	B2G EC (ODec- nil)	IDEXX (S/P ratio)	PM (lesion score)
	Δ bovine (mm)	Δ avian (mm)							
B4	10.2	0	D, O.	0.358	0.237	0.107	0.106	−0.036	1
B10	∞	0	D, N, O.	1.211	1.176	0.758	0.814	0.036	0
B11	∞	0	D, N, O.	0.852	1.169	1.239	0.986	0.609	4
B15	∞	0	D, N, O.	0.455	0.420	0.039	0.034	0.418	0
B25	∞	0	D, O.	0.045	0.034	0.015	0.029	0.155	0
B32	∞	0	D, O.	0.267	0.308	0.083	0.087	0.135	0
B37	6.1	0	D, O.	0.008	0.066	0.026	0.016	1.310	0.5
B41	11.1	0	D, N, O.	0.165	0.143	0.106	0.093	0.662	1
B48	13	0	D, O.	0.150	0.211	0.077	0.092	0.570	1
B53	12.2	0	D, O.	0.266	0.015	0.043	0.026	−0.043	1
B57	∞	0	D, N, O.	0.588	0.042	0.009	0.048	−0.090	2
B75	14.5	0	D, N, O.	0.754	1.172	0.884	1.278	0.108	2
B77	4	0	C.	0.672	0.302	−0.033	−0.028	−0.052	0
B84	10.1	0	D, O.	0.147	0.253	−0.123	−0.128	−0.096	1

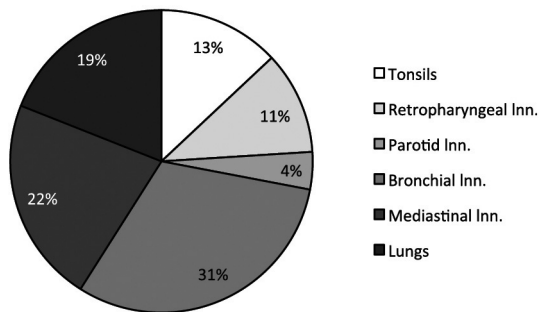


Fig. 1. Post-mortem examination results. The distribution of lesions characteristic of BTB in the lymph nodes and lungs (in percentages).

Overall, the CMI-based assays (SICTT and IFN- γ assays) showed a higher sensitivity than the HI-based assay (IDEXX TB ELISA (37.5%)) (Table 3).

3.6. Parallel application of tests

Relative sensitivity and test agreement were calculated for selected combinations of assays (Table 3) (Petrie and Watson, 2006). The relative sensitivity for in parallel interpretation of test combinations with the SICTT is not shown as this assay had 100% sensitivity in this study due to the bias towards culling SICTT positive buffaloes. The highest sensitivity (100%) was achieved using in parallel testing (Table 3), with any combination of the B1G. A combination of B2G and IDEXX TB ELISA achieved a sensitivity of 75%. Using in series testing the combination of SICTT & B1G assay showed highest sensitivity (100%), while the sensitivity of other test combinations was reduced to either 37.5% or 75%. Test agreement was poor between the IDEXX TB ELISA and all other assays (Table 3).

Table 2b

Results of all assays for several SICTT negative animals, with disparate results. SICTT results are omitted from this table as they were all negative. Results in bold reflect positive results or values above cut-off levels.

Ear tag #	B1G (ODbov-ODav)	B2G A&B (ODbov-ODav)	B2G HP (ODhp-nil)	B2G EC (ODec-nil)	IDEXX (S/P ratio)
B40	0.062	0.019	−0.080	−0.047	0.738
B54	0.020	−0.001	−0.002	0.000	0.470
B65	−0.045	0.002	−0.008	−0.001	0.365
B79	0.025	−0.006	0.002	−0.001	0.307
B83	0.049	0.253	−0.044	−0.044	−0.103
B85	0.012	0.203	−0.065	−0.064	−0.074
B86	0.054	0.309	0.019	0.013	−0.091

Test agreement was good between the B1G and SICTT (0.878), substantial between the B1G and B2G (0.690) and moderate between the B2G and SICTT (0.578) (Table 3).

4. Discussion

The presence of bovine tuberculosis in the Hluhluwe-iMfolozi Park has potentially significant implications for wildlife species in the park, some of which are either vulnerable or endangered, and in addition poses a risk to the livestock and people on neighbouring (communal) farms (Michel et al., 2006). In order to control this devastating disease there is a dire need for accurate diagnostics. Diagnostics for bovine tuberculosis in wildlife, however, are faced with tremendous difficulties in terms of practicality, costs and accuracy (Michel et al., 2011). The SICTT is the most widely used diagnostic tool for bovine tuberculosis in African buffaloes, but more recently IFN- γ release assays have been applied increasingly (Michel et al., 2011). However, the test performances of these assays, single and in combination have rarely been compared in an attempt to develop a diagnostic algorithm for BTB in buffaloes (Grobler et al., 2002, Goosen et al., 2014). In an endemically infected ecosystem such as the HiP, the quest for test fitness for the intended purpose tips the fine balance between sensitivity and specificity, towards sensitivity. In the light of the HiP buffalo BTB monitoring and control program, where SICTT positive animals are culled in an attempt to profoundly decrease the prevalence, a potential overkill i.e. the culling of false positive reactor animals, is considered an acceptable cost for the benefit of optimum test sensitivity and a reduction in the risk of BTB transmission within and between host species. Le Roex et al. (2015), evaluated the success of the test-and-cull program in the HiP and found that although it has been effective at reducing the prevalence of BTB in the park, especially in areas where testing has happened at regular intervals, the

Table 3
Positive responder frequency of the SICTT and relative sensitivity of single and combined application of assays and test agreement between assays. Sensitivity (%) was calculated as the proportion of true positives identified by the test as positive.

Sensitivity (%)	SICTT	B1G	B2G			TB ELISA
			PPD-A/PPD-B	PC-EC ^a	PC-HP ^a	
Single	100	100	75	37.5	50	37.5
In parallel	SICTT & B1G	SICTT & B2G	SICTT & TB ELISA	B1G & B2G	B1G & TB ELISA	B2G & TB ELISA
In series	n/a	n/a	n/a	100	100	75
κ	100	75	37.5	75	37.5	37.5
	0.878	0.578	0.177	0.690	0.123	0.087

^a When both peptide cocktails were used in parallel for stimulation the sensitivity was 50%.

program needs to be optimized further in order to achieve a more pronounced reduction in prevalence whilst preserving population viability. This study aimed at comparison of the test performances of single or combined CMI- and HI-based assays and development of an apt diagnostic protocol for BTB in African buffaloes applicable in the field.

All assays used in this study are commercially available and have been validated for use in cattle. The OIE recognizes that the diagnostic test validation in wildlife is constrained by difficulties such as accessibility to samples, poor sample quality, lack of species-specific reagents, or financial restrictions (World Organisation for Animal Health, 2014). The OIE guideline for validation of diagnostic tests for infectious diseases applicable to wildlife (Chapter 3.6.7 of the Validation Guideline of the Terrestrial Manual) therefore proposes that diagnostic assays validated in domestic animal species can be validated and provisionally recognised in closely related wildlife species using a small sample size (World Organisation for Animal Health, 2014), as applied in this study.

Taking into consideration the limited sample size, this study has shown that the B1G assay showed an apparent superior sensitivity to the B2G assay at 100% compared to 75%, respectively. Previous work on validation of the B1G assay in African buffaloes used a triple comparative stimulation approach, to increase specificity by the inclusion of PPD-F and reported a sensitivity of 86.5% (Michel et al., 2011). In contrast, in the present study the B1G assay was interpreted according to the manufacturer's protocol to achieve highest sensitivity. It should be noted that the sensitivity of the SICTT was most likely overestimated in this study due to the fact that only SICTT positive animals were culled. The sensitivity of the SICTT applied to African buffaloes in the same setting was previously determined to be 80.9% (Jolles et al., 2005). Assuming the sensitivity in the current study was similar or the same, the test performance of the SICTT could possibly be lower than that of the B1G assay. The B2G assay using only the peptide cocktails is not well suited for the testing strategy in the HiP as it shows a lower sensitivity than the SICTT and both IFN- γ assays using the PPDs. The B2G using the PPDs, although showing a lower sensitivity compared to the B1G due to the small sample size in this study, yielded positive results for 3 animals that were negative on all other assays. Their true infection status remained unknown but the potential misclassification of those animals by other tests cannot be ruled out.

Several animals that were not part of the true positive panel tested positive on one or more of the immunoassays performed in this study. Four animals tested positive on both BOVIGAM[®] assays as well as the SICTT, while they did not present with lesions on PM and were thus deemed TB negative. These animals can either have been in early stages of infection, which would explain the lack of lesions, or alternatively, be false positive reactors creating an under- or overestimation of the assay's sensitivity, respectively. The selection bias created by culling SICTT positive animals only, as well as classifying no visible lesions (NVL) reactors as TB negative, was identified as a limitation in this study responsible for the sensitivity to be either over- or underestimated.

The SICTT has several limitations, as it requires boma confinement of the animals for a minimum of 72 h and the animals need to be handled and immobilized twice. Since this is a very costly procedure and bears a risk to the well-being and life of the animals, combined testing with the BOVIGAM[®] assay and the IDEXX TB ELISA, might offer a more cost-effective and sensitive diagnostic alternative, requiring a once-off immobilization only.

To maximize sensitivity of immunodiagnosis of *M. bovis* infection in buffaloes we recommend that the testing strategy includes the parallel use of the BOVIGAM[®] 1G assay as this assay has shown to have a relative sensitivity of 100% in this study. Combination of the B1G assay with the IDEXX TB ELISA assay is likely to increase chances of detection of infected animals in disparate stages of disease and could be fit for the test purpose in HiP. Although the specificity of this serological assay has not been assessed in African buffaloes, a preliminary validation in a small number of sera from known BTB-negative buffaloes showed that it was 100% specific (van der Heijden, unpublished data). This suggests that the four animals testing positive only in the IDEXX TB ELISA might have represented anergic reactors, which were no longer capable of mounting a CMI-response. This could not be confirmed, as these animals were not culled.

In addition the results of the current study justify further investigation of the test performance of the SICTT, BOVIGAM[®] and IDEXX TB ELISA aiming at full validation of these assays. In such a follow-up study, in order to avoid a selection bias, not only SICTT positive, but also SICTT negative yet BOVIGAM[®]- or IDEXX TB ELISA-positive animals should be culled and subjected to a thorough post-mortem examination in order to evaluate the true sensitivity of both the BOVIGAM[®], the IDEXX TB ELISA and the SICTT.

Conflict of interest

none

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