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Mycobacterium bovis prevalence affects the performance of a commercial serological assay for bovine tuberculosis in African buffaloes

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ABSTRACT

The endemic presence of bovine tuberculosis (BTB) in African buffaloes in South Africa has severe consequences for BTB control in domestic cattle, buffalo ranching and wildlife conservation, and poses a potential risk to public health. This study determined the BTB prevalence in free-ranging buffaloes in two game reserves and assessed the influence of the prevalence of mycobacterial infections on the performance of a commercial cattle-specific serological assay for BTB (TB ELISA). Buffaloes (n = 997) were tested with the tuberculin skin test and TB ELISA; a subset (n = 119) was tested longitudinally. Culture, PCR and sequencing were used to confirm infection with *M. bovis* and/or non-tuberculous mycobacteria (NTM). Prevalence of BTB, but not NTM, influenced the TB ELISA performance. Multiple testing did not increase test confidence. The findings strongly illustrate the need for development of novel assays that can supplement existing assays for a more comprehensive testing scheme for BTB in African buffaloes.

1. Introduction

Bovine tuberculosis (BTB) is a chronic infectious respiratory disease caused by *Mycobacterium bovis* (*M. bovis*), affecting a wide range of mammalian hosts [1]. The presence of BTB in several signature wildlife species, both free-ranging as well as captive, has been recognized in different regions of the world for many years [2–6]. The implications of widespread presence of these wildlife reservoirs are far-reaching: BTB eradication efforts in domestic animals are compromised [7,8], the conservation of endangered species may be threatened by further declining populations [9,10] and stifled by movement restrictions, and the wildlife and livestock trade suffer financial losses and face embargos [3,11]. In South Africa in particular, where BTB occurs in domestic cattle and is endemic in some populations of African buffaloes [4,9], where a vast wildlife-livestock-human interface exists, the burden of BTB is of great veterinary and also public health concern [7,12,13]. As a result, there is a need for reliable detection systems for BTB in wildlife.

The complex pathogenesis of the disease urges for accurate detection of both early and chronic stages of BTB, but also renders diagnosis challenging. It is usually based on the detection of the cell-mediated immune (CMI) response to infection with *M. bovis*, as assessed by the tuberculin skin test (TST) or an interferon-gamma release assay (IGRA) such as the BOVIGAM® test [14–16]. These tests were originally designed and validated for use in cattle and although the test performance of both assays is known to be variable [14,15], the TST has proven instrumental in BTB control, and even eradication in cattle, in numerous countries in the past century [17]. In buffaloes, the TST and BOVIGAM® assay have also been widely researched [18–20] and form

Abbreviations: BTB, bovine tuberculosis; CMI, cell-mediated immunity; DAFF, Department of Agriculture, Forestry and Fisheries; HI, humoral immunity; HiP, Hluhluwe-iMfolozi Park; IGRA, interferon-gamma release assay; MGR, Madikwe Game Reserve; MTBC, *Mycobacterium tuberculosis* complex; NTM, non-tuberculous mycobacteria; NVL, no visible lesions; RGM, rapid-growing mycobacteria; SeN, sensitivity of the TB ELISA; SeR, sensitivity of the TST; SFT, skin fold thickness; SGM, slow-growing mycobacteria; SpN, specificity of the TB ELISA; SpR, specificity of the TST; TB ELISA, IDEXX *M. bovis* Ab test (IDEXX Laboratories Inc., US); TST, tuberculin skin test

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part of the national control scheme in South Africa [21,22]. In wildlife, however, control strategies are faced with additional logistical challenges such as the need for two immobilizations and boma-confinement of the animal or limited access to laboratories as required for the respective assays [18,23]. Considering the relative ease of sample collection, serological assays offer a more practical and cost-effective alternative to CMI-testing [23–25]. Additionally, antibody-based diagnostic assays for BTB may allow for detection of anergic animals which otherwise remain undetected [15]. Nevertheless, they are employed less frequently than CMI-based tests, mostly due to low sensitivity and highly variable overall test performance. Geographical location, stage of infection and exposure to and diversity of non-tuberculous mycobacteria (NTM) have been associated with this inconsistent test accuracy [13,24–27]. The precise role of these factors is not well-understood. In South Africa, however, NTM are known to have a widespread distribution and have previously been isolated from buffaloes and their environments [28]. Proteomic analysis of the four most prevalent NTM species revealed the presence of several immunogenic antigens of *M. bovis*, which may be responsible for cross-reactive immune responses [29].

Despite the fact that the sensitivity (Se) and specificity (Sp) of diagnostic tests are traditionally believed to be intrinsic characteristics [30–32], more recent evidence suggests that they may also be influenced by prevalence of disease [33], as is known to be the case for the positive and negative predictive value, and likelihood ratios [30,31,34], which may thus have direct implications for optimum test application. Longitudinal data on the performance of diagnostic tests for BTB in wildlife are very scarce [35–37], yet they can provide valuable information on the usefulness of an assay through multiple application as it may increase the chance of detecting an infected individual as well as boost confidence in consecutive negative results, and thus provide additional information of the accuracy of a given test.

The purpose of the current study was to first determine the prevalence of BTB of buffaloes in the Hluhluwe-iMfolozi Park (HiP) and the Madikwe Game Reserve (MGR), and to subsequently investigate the potential influence of BTB prevalence and occurrence and diversity of NTM on the performance of a commercially available antibody ELISA for the detection of *Mycobacterium bovis* infections (TB ELISA). In addition, in the MGR, multiple testing over a 26-month period was performed to further assess the test performance of the TB ELISA.

2. Materials & methods

2.1. Ethics and regulatory approval

Ethical approval was obtained from the Animal Ethics Committee of the University of Pretoria, under project numbers V050-16 and V138-16, in accordance with the South African National Standard 10386 “The Care and Use of Animals for Scientific Purposes”. A permit under Section 20 of the Animal Diseases Act 1984 (Act no 35 of 84) was obtained from the Directorate Animal Health of the Department of Agriculture, Forestry and Fisheries (DAFF) of the Republic of South Africa.

2.2. Buffalo sampling

2.2.1. Hluhluwe-iMfolozi Park

The Hluhluwe-iMfolozi Park is one of the oldest game reserves in Africa and is located in the province of KwaZulu-Natal in South Africa [38]. The HiP is known to be a BTB endemic area and a BTB management programme was established in 1999 [39]. During 2015-2017, a total of 766 buffaloes comprising 7 herds (A-H) were mass captured and skin tested, as previously described [19]. In 2015, the Masinda section on the iMfolozi side of the park (GPS coordinates 28°15’06”S 31°56’33”E) and in 2016, the Corridor area, on the Hluhluwe side (GPS coordinates 28°13’09”S 32°00’19”E) were targeted. Lastly, in 2017, the Nselweni area on the iMfolozi side of the park was targeted (GPS coordinates 28°18’06.2”S 31°53’29.5”E). Based on previous studies, the BTB prevalence was expected to be high in Masinda and Nselweni, but low in the Corridor area [40].

2.2.2. Madikwe Game Reserve

The Madikwe Game Reserve, founded in 1991, is the country’s fifth largest game reserve (75,000 ha), situated in the North West province of South Africa [41]. Bovine TB was first detected in the Madikwe buffaloes in 2012 [42] and the first interventions in an attempt to curtail BTB took place in 2016. A BTB management proposal was drafted by the North West Parks Board and approved by government, describing the BTB surveillance strategy for the park as well as a buffalo salvage plan aimed at establishing a disease-free buffalo breeding herd outside of Madikwe, given the high value of the ‘Madikwe buffalo brand’. Briefly, a total of 231 buffaloes were captured, using a targeted approach across all sectors of the park, and transported to a temporary holding boma in the Madikwe Game Reserve (general GPS coordinates for the park are 24°76’04”S 26°27’77”E) in early May 2016. Subsequent to initial testing, test positive animals were culled and 143 test negative animals were transported to a permanent boma in the MGR (GPS coordinates 24°45'37"S 26°16'35"E). During the acclimatization period 14 animals broke out, 9 animals died in the boma and 1 animal was culled as it tested positive for brucellosis. A total of 119 animals were enrolled into the longitudinal testing scheme and were kept in the permanent boma for the duration of the study. In 8 subsequent rounds over a period of approximately two years animals were re-tested in September 2016, January 2017, April 2017, August 2017, November 2017, February 2018 and July 2018. In August 2017, 12 animals that were born in the boma were enrolled into the programme.

2.3. Blood collection

Blood was collected from the jugular vein of immobilized animals, either in serum or heparin tubes using a vacutainer system (BD, South Africa) or collected using a 50 mL syringe without anti-coagulant and immediately transferred to serum and heparin vacutainer blood tubes. After clotting, serum tubes were centrifuged at 1500 x *g* for 10 minutes, and sera were harvested and immediately assessed in the TB ELISA. Heparinized blood was processed for and used in the BOVIGAM® assay within 8 hours. Laboratory assays were carried out on site in a field lab in the HiP and the initial round of testing in the MGR (thereafter blood was transported to the Department of Veterinary Tropical Diseases of the University of Pretoria for analysis).

2.4. Tuberculin skin test

The TST was carried out in accordance with OIE standards [43] by officials of the Hluhluwe and Zeerust state veterinary offices. The interpretation of the TST and classification of animals as positive, suspect or negative was performed in both parks as previously described [19]: the test was considered positive when the skin fold thickness (SFT) increase at the bovine site was > 4 mm and the difference in SFT increase between the bovine and avian injection sites was ≥ 2 mm; suspect when the SFT increase at the bovine site was > 4 mm and the difference in SFT increase between the bovine and avian injection sites was 1-2 mm; and negative when the difference between the sites was < 1 mm.

In the HiP, only animals which showed clinical signs at the injection site as assessed by visual inspection from an elevated platform above the holding facility or that tested positive on other assays [44], were immobilized to read and evaluate the TST results. Clinical signs associated with a delayed-type hypersensitivity reaction, indicative of possible *M. bovis* infection, include edematous swelling, exudation, necrosis, tenderness and/or heat of the injected area with or without involvement of the draining lymph node [43]. Due to financial

constraints, animals that showed no obvious clinical signs and tested negative on other assays were not immobilized a second time but assumed negative.

2.5. BOVIGAM® assay

During the first round of testing in the Madikwe Game Reserve the BOVIGAM® assay (Thermo Fisher Scientific, US) was carried out on site in a field lab as previously described [18,19]. Supernatants of stimulated whole blood cultures were harvested and used in the BOVIGAM® assay as per the manufacturer's protocol. Interpretation was carried out according to Michel et al. [18].

2.6. IDEXX M. bovis Ab test (TB ELISA)

The TB ELISA (IDEXX Laboratories Inc., US), a test developed for the detection of *M. bovis* specific antibodies in cattle, was carried out as per the manufacturer's protocol. The only discrepancy to the protocol was that buffalo sera were used undiluted as per previous optimization in this species ([19] and unpublished data).

2.7. Post mortem: bacterial culture, multiplex speciation PCR and sequencing

Animals positive in the TST were culled, as advised by DAFF [22]. Additionally, any animals that got injured, failed to adapt to boma conditions or were not deemed suitable for the salvage plan were culled according to management decisions. All buffalo carcasses were subjected to a detailed post-mortem investigation as previously described [19,45], and tissues collected were processed according to standard operating procedures for mycobacterial culture [46]. In the MGR, nasal swabs collected from 10 randomly selected animals, were processed for mycobacterial culture as previously described [28]. Cultures were monitored for a period of 12 weeks. Crude DNA was extracted from isolates that were identified as rod-shaped and acid-fast, using Ziehl-Neelsen staining. A multiplex PCR was used for the detection of *Mycobacterium* spp. and differentiation of MTBC species, as previously described [47]. Samples which tested positive for the genus, but could not be differentiated further, were amplified in a 16S ribosomal RNA PCR [48]. Amplicons were sequenced at Inqaba Biotechnical Industries (Pretoria, South Africa) and sequences obtained analyzed using the NCBI Nucleotide Basic Local Alignment Search Tool (BLAST) [49] for species identification. Alignments and phylogenetic analysis were performed using the CLC Main Workbench (Qiagen Bioinformatics, Aarhus, Denmark). Reference sequences of *M. bovis, M. asiaticum* and *M. moriokaense* were retrieved from GenBank [50] and included as an outgroup species, and representatives of slow- (SGM) and rapid-growing mycobacteria (RGM), respectively. The phylogenetic tree was constructed using the neighbor-joining method with 1,000 bootstrap replicates and validated using the maximum composite likelihood method.

2.8. Data analysis

Given the lack of a true gold standard test, the TST was used as the reference test to which the test performance of the TB ELISA was related [51]. The Se of the TST in African buffaloes was estimated to be 76.5%, while the Sp was estimated to be 99.5%, based on published and unpublished data from the authors [18]. In order to model the uncertainty in these estimates, a beta-distribution of the Se and Sp of the TST (SeR and SpR, respectively) was made using R [52,53]. Calculations of BTB prevalence (animal-level) and test performance of the TB ELISA were based on formulas derived from Staquet et al. [51]. Prevalence (P) was calculated using the formula: ((n * (SpR - 1) + TP + FN) / (n * (SeR + SpR -1))) * 100; where n = total number of animals, TP = true positives and FN = false negatives. The Se of the TB ELISA (SeN) was calculated using the formula: (((TP + FP) * SpR - FP) / (n * (SpR - 1) + TP + FN)) * 100, where FP = false positives. The specificity of the TB ELISA (SpN) was calculated using the formula: (((FN + TN) * SeR - FN) / ((n * SeR) - (TP + FN))) * 100, where TN = true negatives. The positive predictive value (PPV) of the TB ELISA was calculated using the formula: (SeN * (TP + FN - n + SpR * n)) / (TP + FP * (SeR + SpR - 1)). The negative predictive value (NPV) of the TB ELISA was calculated using the formula: (SpN * (n * SeR - TP - FN)) / ((SeR + SpR - 1) * (FN + TN)). The 95% confidence intervals (95% CI) for Se, Sp, PPV and NPV were determined using the Stats package of R by calculating the 2.5% and 97.5% quantiles of the calculated beta-distributions [53,54]. Given that the study was conducted in infected herds, estimates for the SpN and NPV are approximate values. Based on previous work in HiP [39,40], a prevalence of < 15% was classified as low, whereas a prevalence of ≥15% was classified as high.

3. Results

3.1. Determination of BTB prevalence

During 2015-2017, a total of 766 buffaloes comprising 7 herds (A-H) were captured in the three targeted areas of the HiP and tested by the TST. A summary of the results of the HiP testing can be found in Fig. 1A and Table 1. All TST positive animals were culled. For analyses, data of animals in herds of the HiP were subsequently grouped together based on the BTB prevalences found, to form a low prevalence cohort (herds C-G) and a high prevalence cohort (herds A, B and H) (Table 2).

In May 2016, a total of 231 buffaloes comprising 6 herds (1-6) were captured in the MGR and tested by TST. A summary of the results is shown in Fig. 1B and Table 1. All TST positive animals were culled. For analyses, data of animals in herds of the MGR were subsequently grouped together based on BTB prevalences found, to form a low prevalence cohort (herds 3 and 5) and a high prevalence cohort (herds 1, 2, 4 and 6) (Table 2).

3.2. Results of the BOVIGAM® assay

In the MGR, the BOVIGAM® assay was carried out in parallel to the TST during the initial testing round. A total of 31/231 (13.4%) animals were excluded from the analysis as the internal positive control (PWM) of the assay was invalid. Additionally, the blood sample of one animal was lacking. In total, 35/199 (17.6%) animals tested positive in the BOVIGAM® assay. The majority of these animals (28/35) was also positive in the TST (TST/BOVIGAM positive). Two of the 7 TST negative, BOVIGAM positive animals were also positive in the TB ELISA (BOVIGAM/ELISA positive), while the other five were exclusively positive in the BOVIGAM® assay.

3.3. Results and test performance of the TB ELISA

In the HiP, 101/766 (13.2%) animals tested positive in the TB ELISA. Of the TB ELISA positive animals, 14/101 (13.9%) were also TST positive (TST/ELISA positive). The remaining 87/101 (86.1%) were exclusively positive in the TB ELISA (TB ELISA positive). In the MGR, 48/230 (20.9%) animals tested positive in the TB ELISA. Of the TB ELISA positive animals, 2/48 (4.2%) were TST/ELISA positives, 2/48 (4.2%) were BOVIGAM/ELISA positive, 7/48 (14.6%) were positive in all three assays and the remaining 37/48 (77.1%) were exclusively positive in the TB ELISA.

The test performances of the TB ELISA in the low and high prevalence cohorts of the HiP and the MGR are presented in Table 2. In the HiP, the overall BTB prevalence in the low prevalence cohort was 7.7% (95% CI = 6.8% - 8.6%), while that in the high prevalence cohort was 24.0% (95%CI = 22.1% - 26.3%). The SeN was significantly lower in the low prevalence cohort (0%; 95% CI = n/a) as compared to the high prevalence cohort (27.4%; 95% CI = 27.4 - 27.5%). The SpN, however,

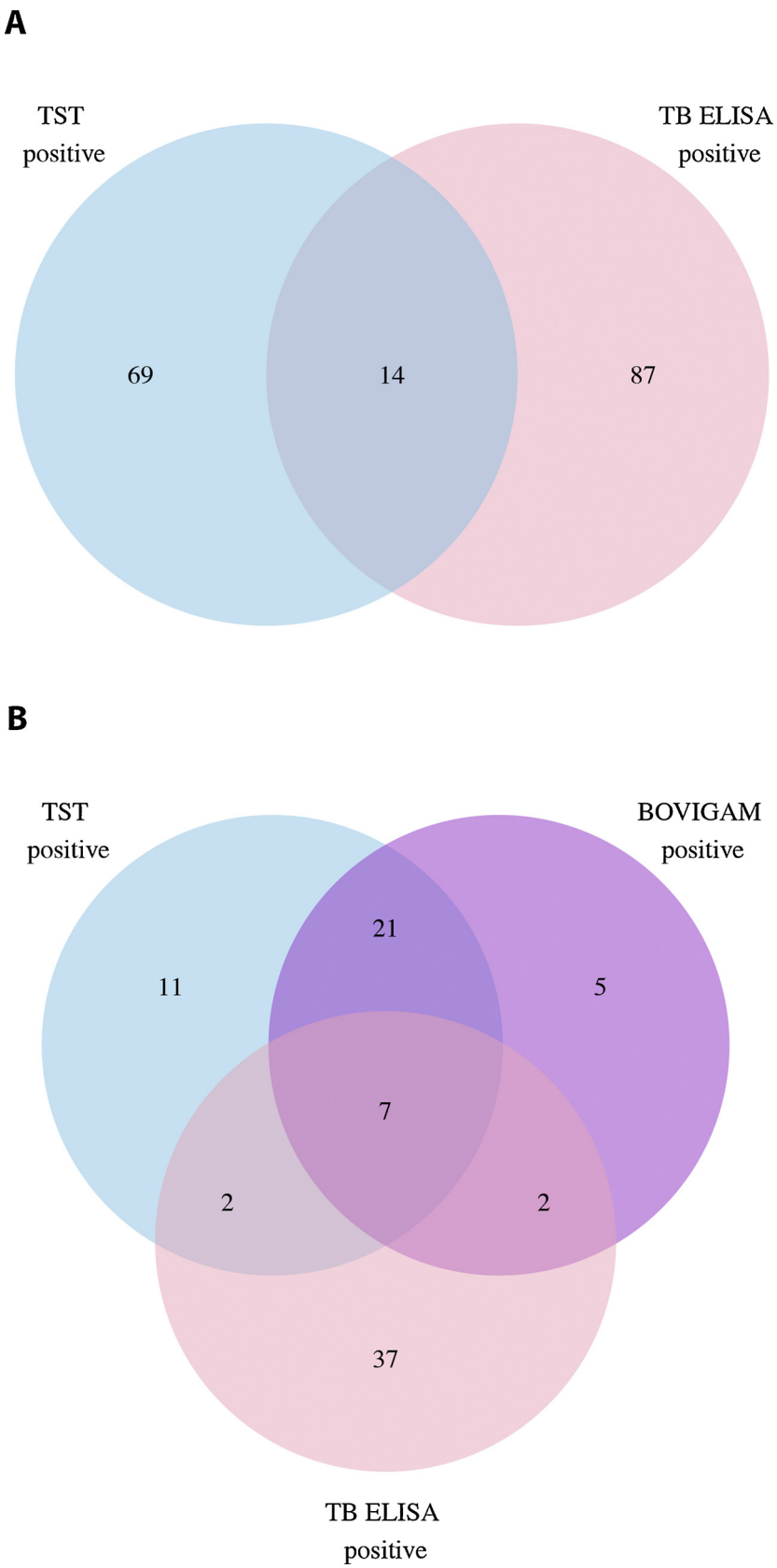

Fig. 1. Venn diagram reflecting test results of HiP and MGR. Results of the TST, BOVIGAM® assay and TB ELISA for the BTB testing programme (A) in the HiP during 2015-2017 and (B) in the MGR in May 2016. Only positive reactors are shown. A total of 596 and 141 buffaloes tested negative on all assays in the HiP and MGR, respectively (data not shown). Created using the VennDiagram package version 1.6.20 [76] in R [53].

Table 1
BTB prevalence in the HiP and MGR. The prevalence of BTB in herds in the HiP and MGR was calculated according to Staquet et al. [51] based on an estimated TST Se of 76.5% and Sp of 99.5%. Estimates and 95% confidence intervals (CI) are given for each herd in both reserves as well as an overall estimate for each reserve. HiP = Hluhluwe iMfolozi Park; MGR = Madikwe Game Reserve.

HiP	Area and year	Herd	Test positive animals (positive/sampled)	Prevalence Estimate (%)	95% CI (%)
	Masinda 2015	A	18/106 (17.0%)	21.6	19.9 - 23.7
		B	18/91 (19.8%)	25.4	23.4 - 27.7
		C	8/86 (9.3%)	11.5	10.4 - 12.7
	Corridor 2016	D	8/182 (4.4%)	5.0	4.1 - 5.7
		E	9/150 (6.0%)	7.1	6.1 - 8.0
	Nselweni 2017	F	3/36 (8.3%)	10.2	9.1 - 11.3
		G	4/40 (10%)	12.4	11.2 - 13.7
		H	15/75 (20.0%)	25.6	23.6 - 28.1
	Overall		83/766 (10.8%)	13.5	12.3 - 14.9
MGR	Area and year	Herd	Test positive animals (positive/sampled)	Prevalence Estimate (%)	95% CI (%)
	Entire park May 2016	1	6/22 (27.3%)	35.3	32.6 - 38.6
		2	7/31 (22.6%)	29.1	26.8 - 31.8
		3	2/43 (4.7%)	5.3	4.4 - 6.1
		4	6/49 (12.2%)	15.4	14.0 - 16.9
		5	3/41 (7.3%)	8.8	7.8 - 9.8
		6	17/45 (37.8%)	49.2	45.5 - 53.7
	Overall		41/231 (17.7%)	22.7	20.9 - 24.8

was significantly higher in the low prevalence cohort (94.7%; 95% CI = 94.6% - 94.7%) as compared to the high prevalence cohort (71.9%; 95% CI = 71.9 - 71.9%). The estimates of the PPV and NPV followed the same trend as the SeN and SpN, respectively (Table 2). In the MGR, the prevalence in the low prevalence cohort was 5.4% (95% CI = 4.5% - 6.2%), while that in the high prevalence cohort was 26.2% (95% CI = 24.1% - 28.6%). In the MGR, the SeN showed an inverse relationship to the prevalence as compared to that in the HiP: the SeN was significantly higher in the low prevalence cohort (54.6%; 95% CI = 51.3% - 60.8%) as compared to the high prevalence cohort (17.9%; 95% CI = 17.8% - 17.9%). Similar to what was observed in the HiP, the SpN was significantly higher in the low prevalence cohort (80.2%; 95% CI = 80.1% - 80.4%) as compared to the high prevalence cohort (78.3%; 95% CI = 78.1% - 78.4%) in the MGR. While, the NPV followed the same trend as the SpN in the MGR, the PPV did not follow that of the SeN (Table 2): the PPV was significantly lower in the low prevalence cohort (0.14; 95% CI = 0.13 - 0.15) as compared to the high prevalence cohort (0.30; 95% CI = 0.28 - 0.33). The NPV was significantly higher in the low prevalence cohort (0.97; 95% CI = 0.96 - 0.98) as compared to the high prevalence cohort (0.65; 95% CI = 0.61 - 0.67).

3.4. Mycobacterial isolation

During the post-mortem investigations the spectrum of pathological changes was wide, varying from no visible lesions (NVL) to pinpoint lesions in one to two organs, to extensive lesions in the lungs and lymph nodes (LNN). One animal in the MGR presented with miliary tuberculosis (Fig. S1). The results of mycobacterial isolation from low and high prevalence cohorts in the HiP and the initial isolation in the MGR are presented in Table 2.

In the low prevalence cohort of the HiP, a total of 32 TST positive animals and 11 TB ELISA positive animals were culled (n = 43), of which 38 were sampled. No samples were obtained from 4 TST positive animals and 1 TB ELISA positive animal. *Mycobacterium bovis* infection was confirmed in 12/28 (42.9%) TST positive animals and 0/10 (0%) TB ELISA positive animals. Infection with NTM was confirmed in 7/28 (25.0%) TST positive animals and 3/10 (30.0%) TB ELISA positive animals. In the high prevalence cohort of the HiP, a total of 37 TST positive animals, 14 TST/ELISA positive animals, and 7 TB ELISA positive animals were culled (n = 58). Tissue samples from 46 animals were processed but no samples were obtained from 10 TST positive animals, 1 TST/ELISA positive animal, and 1 TB ELISA positive animal. *Mycobacterium bovis* infection was confirmed in 23/27 (85.2%) TST positive animals, 8/13 (61.5%) TST/ELISA positive animals, and 1/6 (16.7%) TB ELISA positive animals. Infection with NTM was confirmed in 2/27 (7.4%) TST positive animals, 1/13 (7.7%) TST/ELISA positive animals, and 1/6 (16.7%) TB ELISA positive animals.

In the low prevalence cohort of the MGR, a total of 2 animals positive in all three assays, 2 TST positive animals, 1 TST/BOVIGAM positive animal, and 1 BOVIGAM positive animal were culled (n = 6). *Mycobacterium bovis* infection was confirmed in 2/2 (100%) animals positive in all three assays, 1/2 (50%) TST positive animals and 1/1 (100%) TST/BOVIGAM positive animal. In the high prevalence cohort of the MGR, a total of 5 animals positive in all three assays, 8 TST positive animals, 2 TST/ELISA positive animals, 20 TST/BOVIGAM positive animals, 5 TB ELISA positive animals, 2 BOVIGAM/ELISA positive animals, 4 BOVIGAM positive animals and 8 test negative animals (n = 54) were culled. *Mycobacterium bovis* infection was confirmed in 3/5 (60%) animals positive in all three assays, 5/8 (62.5%) TST positive animals, 2/2 (100%) TST/ELISA positive animals, 8/20 (40%) TST/BOVIGAM positive animals, 2/5 (40%) TB ELISA positive animals, and 2/4 (50%) BOVIGAM positive animals.

Of the collected nasal swab samples 6 were collected from animals in the low prevalence cohort of the MGR, belonging to 3 TB ELISA positive animals and 3 test negative animals. Non-tuberculous

Table 2
Prevalence of BTB, performance of TB ELISA and mycobacterial isolation in low and high prevalence cohorts in the HiP and the MGR. Prevalence of BTB was calculated according to Staquet et al. [51] based on an estimated TST Se of 76.5% and Sp of 99.5%. Estimates and 95% confidence intervals (CI) are given for each cohort in both reserves. HiP = Hluhluwe iMfolozi Park; MGR = Madikwe Game Reserve; *M. bovis* = *Mycobacterium bovis*; NPV = negative predictive value; NTM = non-tuberculous mycobacteria; PPV = positive predictive value; P = prevalence; p = proportion; positive/sampled = the ratio of the number of *M. bovis* or NTM positive samples out of the total number of samples processed for culture; SeN = sensitivity of the TB ELISA; SpN = specificity of the TB ELISA; † = mycobacterial isolation from tissue samples; * = mycobacterial isolation from nasal swabs.

Location	Cohort	Prevalence		TB ELISA test performance								Mycobacterial isolation	
		Estimate (%)	95% CI (%)	SeN (%)	95% CI (%)	PPV (p)	95% CI (p)	SpN (%)	95% CI (%)	NPV (p)	95% CI (p)	*M. bovis (positive/ sampled)*	NTM *(positive/ sampled)*
HiP	Low P	7.7	6.8 - 8.6	0	n/a	0	n/a	94.7	94.6 - 94.7	0.92	0.91 - 0.93	12/38 (31.6%) †	10/38 (26.3%) †
	High P	24.0	22.1 - 26.3	27.4	27.4 - 27.5	0.24	0.22 - 0.26	71.9	71.9 - 71.9	0.76	0.74 - 0.78	32/46 (69.6%) †	4/46 (8.7%) †
MGR	Low P	5.4	4.5 - 6.2	54.6	51.3 - 60.8	0.14	0.13 - 0.15	80.2	80.1 - 80.4	0.97	0.96 - 0.98	4/6 (33.3%) † 0/6 (0.0%) *	0/6 (0.0%) † 4/6 (33.3%) *
	High P	26.2	24.1 - 28.6	17.9	17.8 - 17.9	0.30	0.28 - 0.33	78.3	78.1 - 78.4	0.65	0.61 - 0.67	22/54 (40.7%) † 0/4 (0.0%) *	0/54 (0.0%) † 2/4 (50.0%) *

mycobacteria were isolated from 2/3 TB ELISA positive animals and 2/3 test negative animals. The other 4 nasal swab samples were collected from animals in the high prevalence cohort of the MGR, belonging to 2 TB ELISA positive animals and 2 test negative animals. Non-tuberculous mycobacteria were isolated from the 1/2 TB ELISA positive animals and 1/2 test negative animals.

3.5. Longitudinal testing

For the longitudinal testing in the MGR, animals were re-tested in September 2016, January 2017, April 2017, August 2017, November 2017, February 2018 and July 2018. A summary of the results of the longitudinal testing in the MGR is shown in Table 3.

3.5.1. Determination of BTB prevalence and mycobacterial isolation

An overall trend of declining BTB prevalence with subsequent testing rounds and removal of reactor animals was observed (Table 3). In September 2016, 11/119 (9.2%) animals tested positive in the TST, and the BTB prevalence was low at 11.7% (95% CI = 10.6% - 12.9%). The TST positive animals and an additional 26 animals were removed, but the process did not allow for post mortem (PM) investigation of the carcasses. A further 5 animals died in the boma during this period. In January 2017, 0/67 (0%) animals tested positive in the TST, and the BTB prevalence was reduced to 0% (95% CI = n/a). Eight test negative animals were removed of which opportunistic PM investigation and sampling was carried out and NTM were isolated from 4/8 (50%) animals (Table 3). Five animals died in the boma during this period. From April 2017 until the end of the study, no animals tested positive in the TST (Table 3) and the BTB prevalence remained 0% (95% CI = n/a). In August 2017 one animal was removed, another animal was removed in November 2017 and in February 2018 seven animals were removed of which opportunistic PM investigation and sampling were carried out. Infection with *Mycobacterium bovis* was confirmed in the animal removed in November 2017 and NTM were isolated from 1/7 (14.3%) animals removed in February 2018 (Table 3).

3.5.2. Results and test performance of the TB ELISA

An overall trend of decreasing numbers of test positive animals with subsequent testing rounds was observed, with the exception of February 2018. The proportions of TB ELISA positive animals in each testing round between September 2016 and July 2018 are presented in Table 3. All animals testing positive in the TB ELISA, were negative in the TST. The SeN could not be determined from January 2017 until the end of the study as the estimated BTB prevalence was 0% at each of the sampling occasions during that period. The test performance of the TB ELISA from September 2016 to July 2018 is shown in Table 3. An inverse relationship was observed between the SeN and SpN: the SeN had a median of 0% (range = 0% - 0%), while the SpN had a median of 94.3% (range = 88.8 - 98.4). The PPV of the TB ELISA was 0 (range = n/a) throughout the longitudinal study. The NPV on the other hand had a median of 1 (range = 0.74 - 1).

3.6. 16S ribosomal RNA sequence analysis

In total, DNA samples from bacterial isolates from 3 nasal swabs and 20 tissue samples from the HiP and MGR that were identified as NTM DNA in the multiplex PCR (Figs. S2 and S3), were sequenced. Sequences alignments are shown in Fig. S4. Results of the BLAST analysis [49] are presented in Table 4. In total, 18/23 isolates, could be differentiated to the species level and the following NTM were identified: *M. brasiliensis*, *M. flavescens*, *M. moriokaense*, *M. holsaticum*, *M. agri*, *M. asiaticum*, *M. celatum*, *M. avium* complex and *M. rhodesiae* (Table 4). Other species that were identified in isolates that could not be differentiated to the species level include *M. smegmatis/M. goodii*, *M. moriokaense/M. barrassiae*, *M. colombiense/M. bouchedurhonense* and *M. vulneris/M. intracellulare*. In one animal, two different species of NTM were found, *M. holsaticum* and *M. brasiliensis* (Table 4). The identified species of NTM included both SGM [55–57], and RGM [56–59], as well as species belonging to the *Mycobacterium avium* complex (MAC) [60] and *Mycobacterium moriokaense* group [60,61]. A phylogenetic tree of all isolates is presented in Fig. S5.

4. Discussion

The purpose of the present study was to determine the BTB prevalence in different free-ranging African buffalo herds in two game reserves and to use these data to investigate whether the BTB prevalence affects the performance of a commercially available cattle-specific TB antibody assay (TB ELISA). In addition, the impact of the occurrence and diversity of infections with NTM on the performance of the TB ELISA was evaluated. Finally, the contribution of multiple serological testing on the test performance of the TB ELISA was examined in a longitudinal study.

4.1. BTB prevalence

Overall, the BTB prevalence in the HiP was found to be 13.5% (95% CI = 12.3% - 14.9%) in this study, which was classified as low. Le Roex et al. [40] previously determined that the BTB prevalence (outside disease 'hot spots') in the HiP remained low at 10% - 15% over a period of 7 years (1999 - 2006). In their study, the prevalence of BTB was based on the TST only, without correction for the test performance.

Table 3
The test performance of TB ELISA and mycobacterial isolation results for the longitudinal study of the MGR. Prevalence of BTB was calculated according to Staquet et al. [51], based on an estimated TST Se of 76.5% and Sp of 99.5%. Estimates and 95% confidence intervals (CI) are given for all time points. *M. bovis* = *Mycobacterium bovis*; NPV = negative predictive value; NTM = non-tuberculous mycobacteria; PPV = positive predictive value; P = prevalence; p = proportion; positive/sampled = the ratio of the number of *M. bovis* or NTM positive samples out of the total number of samples processed for culture; SeN = sensitivity of the TB ELISA; SpN = specificity of the TB ELISA; ND = not done. No serum was received from 1 animal in April 2017 and 4 animals in July 2018.

Time point	Prevalence		Proportion of TB ELISA positives (positive / tested)	TB ELISA test performance								Mycobacterial isolation	
	Total (%)	95% CI (%)		SeN (%)	95% CI (%)	PPV (p)	95% CI (p)	SpN (%)	95% CI (%)	NPV (p)	95% CI (p)	*M. bovis* (positive/ sampled)	NTM (positive/ sampled)
Sept 2016	11.7	10.6 - 12.9	5/119 (4.2%)	0	n/a	0	n/a	95.1	95.0 - 95.1	0.88	0.86 - 0.89	ND	ND
Jan 2017	0	n/a	4/67 (6.0%)	n/a	n/a	0	n/a	94.0	94.0 - 94.0	1	n/a	0/8 (0.0%)	4/8 (50.0%)
Apr 2017	0	n/a	3/53 (5.7%)	n/a	n/a	0	n/a	94.3	94.3 - 94.3	1	n/a	n/a	n/a
Aug 2017	0	n/a	1/66 (1.5%)	n/a	n/a	0	n/a	98.5	98.5 - 98.5	1	n/a	0/1 (0.0%)	0/1 (0.0%)
Nov 2017	0	n/a	1/65 (1.5%)	n/a	n/a	0	n/a	98.5	98.5 - 98.5	1	n/a	1/1 (100.0%)	0/1 (0.0%)
Feb 2018	0	n/a	7/64 (10.9%)	n/a	n/a	0	n/a	89.1	89.1 - 89.1	1	n/a	0/7 (0.0%)	1/7 (14.3%)
Jul 2018	0	n/a	1/52 (1.9%)	n/a	n/a	0	n/a	98.1	98.1 - 98.1	1	n/a	n/a	n/a

Considering that the estimated Se of the TST in buffaloes is 76.5%, it is reasonable to assume that the BTB prevalence had in fact been considerably higher at the time of their study. As such, the finding that the overall BTB prevalence was low more than a decade later, confirms that the programme is effective at keeping the disease at bay [40]. Considering that the infection in the HiP is an established, endemic infection, first detected in the park in 1986 in a buffalo [39], but most likely present much longer, this finding is especially pertinent. The fact that the BTB prevalence in the much more recent BTB epidemic in the MGR, where no control measures had taken place up to 2016, was significantly higher (22.7%; 95% CI = 20.9% - 24.8%), corroborates that the approach taken in HiP is effective. Although eradication of BTB from an endemically infected reserve is unlikely to be achieved, it is important to intervene in order to reduce transmission and prevent spill-over into endangered wildlife species or spillback to livestock.

4.2. Test performance of the TB ELISA

The test performance of the TB ELISA was highly variable in the two reserves, but also when assessed in and compared between cohorts of low and high BTB prevalence. The SeN varied between 0% (95% CI = n/a) and 54.6% (95% CI = 51.3% - 60.8%), whilst the SpN varied between 71.9% (95% CI = 71.9% - 71.9%) and 94.7% (95% CI = 94.6% - 94.7%). The PPV of the TB ELISA varied between 0 (95% CI = n/a) and 0.30 (95% CI = 0.28 - 0.33), whilst the NPV varied between 0.65 (95% CI = 0.61 - 0.67) and 1 (95% CI = n/a).

During the course of the longitudinal study the test performance showed less variability, but it is important to note that there were increasingly fewer infected animals in the study due to removal of TST positive animals. The SeN was 0% or uninterpretable during the course of the longitudinal study, but a gradual increase in the SpN was observed over time (Table 3). The PPV and NPV clearly reflect the increasingly lower prevalence in this cohort of animals as the PPV was 0 for the duration of the longitudinal study, while the NPV changed from 0.88 (95% CI = 0.86 - 0.89) in September 2016 to 1 (95% CI = n/a) thereafter.

Of an exceptionally large number of TB ELISA positive animals (negative on other assays) (n = 37) detected in May 2016, 29/30 (96.7%) animals enrolled in the longitudinal study subsequently tested negative 3 months later. Twelve of these animals remained in the study for the entire duration and while 6/12 (50.0%) were consistently negative, 5/12 (41.7%) had erratic positive results and 1/12 (8.3%) was consistently positive, in the TB ELISA. In the TST, all of these animals were consistently negative. True disease status was not assessed in these animals, and it is thus not possible to know for certain whether these animals were true or false positive in the TB ELISA.

4.3. Factors influencing the test performance of the TB ELISA

The observed relationship between BTB prevalence and the PPV and NPV was to be expected as it is inherent to their definitions [31,34]. Sensitivity and specificity, however, are usually not linked to disease prevalence as they are considered attributes of the test [31,32]. In the present study, however, the SeN and SpN were found to vary with prevalence (Table 2). Interestingly, the effect of the BTB prevalence on the SeN and SpN was not unidirectional. In the HiP, low prevalence was associated with a low SeN, but acceptable SpN, while high prevalence was associated with a higher SeN, but lower SpN. The opposite was demonstrated for the SeN in the MGR. This lack of a systematic, unidirectional, effect of prevalence on test performance has been reported previously [33]. Underlying factors can be divided into clinical (i.e. related to the epidemiological setting) versus artefactual variability (i.e. related to study design) [33]. In this study, the fluctuation of test performance may partly be attributed to the latter, given that the calculations of SeR and SpR might have been affected by verification bias [31]. The gold standard test for *M. bovis* diagnosis is culture of tissue

Table 4
Basic Local Alignment Search Tool (BLAST) analysis results. If more than one species was identified with equal query cover and identity, both are listed. TST = tuberculin skin test; MGR = Madikwe Game Reserve; LNN = lymph nodes, NVL = no visible lesions; HiP = Hluhluwe iMfolozi Park; "+" = positive; "- " = negative.

Sample ID	Origin	Immuno-diagnosis (TST / TB ELISA)	Sample description	Isolate morphology	Growth rate (weeks)	Species identified	Accession number
BSL 122	MGR May 2016	- / -	Nasal swab	Dry, mustard-yellow colonies	1	*M. brasiliensis*	EU165538.1
BSL 123	MGR May 2016	- / -	Nasal swab	Dry, mustard- to orange-yellow colonies	1	*M. flavescens*	MH169222.1
BSL 125	MGR May 2016	- / +	Nasal swab	Dry, light-yellow to yellow colonies	1	*M. moriokaense*	AB649000.1
BSL 378	MGR Jan 2017	- / -	Pooled LNN; NVL	Mucoid, light-yellow colonies	3	*M. holsaticum*	AJ310467.1
BSL 386	MGR Jan 2017	- / -	Pooled LNN; NVL	Mucoid, yellow colonies	2	*M. holsaticum*	NR_028945.2
BSL 395	MGR Jan 2017	- / -	Pooled LNN; NVL	Dark-yellow colonies	1	*M. agri*	NR_025527.1
BSL 398	MGR Jan 2017	- / -	Pooled LNN; NVL	Dark-yellow colonies	2	*M. smegmatis*/ *M. goodii*	5O5J_A/ KT597551.1
BSL 629	MGR Feb 2018	-/-	Pooled LNN; NVL	Dry yellow colonies	1	*M. rhodesiae*	AB649001.1
BSL 401	HiP 2016	+ / -	Pooled LNN & tonsils; suspect lesions	Light- to dark-yellow colonies	4	*M. asiaticum*	AY722097.1
BSL 407	HiP 2016	+ / -	Pooled LNN & tonsils; NVL	Dry, cream & mucoid, yellow mixed colonies	2	*M. brasiliensis*	EU165538.1
BSL 447	HiP 2016	+ / -	Pooled LNN; NVL	Dark-yellow colonies	5	*M. asiaticum*	AY722097.1
BSL 450	HiP 2016	- / +	Pooled LNN; one suspect lesion	Dry, cream & mucoid light-yellow mixed colonies	3	*M. brasiliensis*	EU165538.1
BSL 453	HiP 2016	+ / -	Pooled LNN; suspect lesions	Dry, cream to dark-yellow mixed colonies	1	*M. moriokaense*/ *M. barrassiae*	NR_115331.1/ NR_115330.1
BSL 469	HiP 2016	+ / -	Pooled LNN; suspect lesions	Dry, cream to dark-yellow mixed colonies	5	*M. flavescens*	LC082329.1
BSL 474C	HiP 2017	+ / -	Pooled LNN; NVL	Mucoid, cream colonies	1	*M. holsaticum*	AJ310467.1
BSL 474Y	HiP 2017	+ / -	Pooled LNN; NVL	Dry, dark-yellow colonies	2	*M. brasiliensis*	EU165538.1
BSL 480	HiP 2017	- / +	Lung tissue with lesions	Dry & mucoid, light-yellow mixed colonies	3	*M. colombiense*/ *M. bouchedurhonense*	NR_117222.1/ NR_116063.1
BSL 484	HIP 2017	+ / -	Pooled LNN; NVL	Dry, yellow colonies	1	*M. brasiliensis*	EU165538.1
BSL 498	HiP 2017	+ / -	Pooled LNN; NVL	Mucoid, cream to light-yellow colonies	2	*M. holsaticum*	AJ310467.1
BSL 499	HiP 2017	- / +	Pooled LNN; NVL	Dry, light- to dark-yellow colonies	7	*M. moriokaense*/ *M. barrassiae*	NR_115331.1/ NR_115330.1
BSL 502	HiP 2017	+ / +	Pooled LNN; NVL	Dry, cream colonies	10	*M. celatum*	JQ582668.1
BSL 508	HiP 2017	+ / -	Pooled LNN; NVL	Dry, dark-yellow colonies	2	*M. avium* complex	KJ920349.1
BSL 513	HiP 2017	- / +	Pooled LNN; NVL	Dry, cream colonies	6	*M. vulneris*/ *M. intracellulare*	LT718448.1/ AY652956.1

samples collected during post mortem [9], but since the decision to cull is usually based on a positive TST result, confirmation of the disease is often only attempted in a subset of animals biased towards false positives, while ignoring false negatives. This factor could be exacerbated in both very early and exceedingly chronic infections, as the TST is known to have a lower sensitivity in those stages of disease [14]. However, as every attempt was made in this study to estimate the test performance of the TST as accurately as possible, we believe that artefactual variability was kept to a minimum and the findings are most likely a result of the history and epidemiology of these herds. In fact, the considerably disparate endemicity and chronicity of the infection in the two study populations might play a role in this. Another explanation for the variability in performance of the TB ELISA might be exposure to NTM, that is known to interfere with diagnosis of BTB due to eliciting cross-reactive immune responsiveness [28,62]. Cross-reactivity is believed to occur due to immune recognition of mycobacterial antigens that are shared between species of the *Mycobacterium tuberculosis* complex (MTBC) and certain NTM [62]. The occurrence of NTM may vary in geographically distinct areas and a high diversity of NTM has previously been reported in South Africa [26,28]. In the present study, a high and comparable degree of diversity of NTM was detected in buffaloes from the HiP and the MGR. Phylogenetic relatedness of the isolated species of NTM and their closeness to *M. bovis* is depicted in Fig. S5. Most of the species isolated in this study have previously been described to occur in South African wildlife species and their environment [26,28], with the exception of *M. agri*, *M. barrassiae* (although not differentiated to the species level in this study) and *M. celatum*. The species of NTM identified in this study have been linked to (opportunistic) infections in humans [59–61,63], excluding *M. agri* which appears to be associated mainly with soil [64]. The most frequently isolated NTM species was *M. brasiliensis*. Interestingly, the same species was consistently isolated from milk samples from cattle on communal farms surrounding the HiP [65]. In that study, however, the diversity of NTM was very low. Despite the high diversity of NTM in the present study, and the fact that NTM were isolated from several TB ELISA positive animals (Table 4), an effect on the test performance of the TB ELISA could not be demonstrated. It is important to note, however, that due to the low confidence in the TB ELISA, the majority of TB ELISA positive animals were not culled, making it difficult to assess the true disease status of these animals.

Of all isolates, those belonging to the MAC were most closely related to *M. bovis* (Fig. S5). The inclusion of the purified protein derivative of *M. avium* in the TST and BOVIGAM® assay as a representative NTM would thus appear to remain relevant in this setting, as these species would be expected to cause the most interference. Furthermore, while a large proportion of NTM were isolated from animals that were TST positive (10 samples from the HiP) (Table 4), it is unlikely that all these were false positive reactors. Given the estimated Sp of 99.5% for the TST, a maximum of 5 false positive TST reactors would be expected from the total number of buffaloes (n = 997) tested in this study. It is possible that co-infections with *M. bovis* and NTM species were missed in this study, as NTM are known to outcompete slow-growing pathogenic mycobacteria.

Lastly, besides the geographical differences in the distribution of NTM, there might be other factors related to the environment that could impact test performance. Trost et al. [13] postulated that sequence variation in the genes of MPB70 and MPB83 of *M. bovis*, or the genes that regulate their expression, sigK and rskA, might explain geographical differences, as the TB ELISA measures antibody (Ab) levels against proteins encoded by these genes [13]. Although this hypothesis remains to be proven, similar processes could occur in different species of NTM, potentially giving rise to irregularities in test performance.

In a study by Mhongovoyo [66] of the BTB prevalence of a population of buffaloes in Botswana previously believed to be free of BTB [67], all animals tested (n = 60) were positive in the TB ELISA, while the entire cohort was negative in the BOVIGAM® assay. The conclusion was drawn that these animals were in fact false positive, demonstrating an exceptionally poor specificity of the TB ELISA. The underlying reason for this is unknown, but may be (partly) attributed either to cross-reactive responsiveness due to NTM exposure, as was suggested by Mhongovoyo [66]. While this initially seemed plausible in the MGR, due to observed plentiful rains which could have created conditions favorable to NTM [55], it was deemed unlikely to be the sole cause, given that similar results were never repeated despite the study having been conducted over a period of two years. Potential non-specific reactions relating to the test platform might include e.g. i) drug combinations used for the immobilization of these animals; ii) the test kit batch; or iii) sample. The true underlying reason remains unclear but warrants further investigation.

It is furthermore important to note that the humoral immune response in bovine TB, while not fully understood, is thought to commence in later stages of infection [68] and appears to be correlated with pathology in several species [69–71]. As such, disparate stages of infection of the study animals may have contributed to the erratic test performance of the TB ELISA as demonstrated in this study. Considering the variability, these findings show that the TB ELISA is of limited diagnostic value in free-ranging buffalo populations and is not recommended for use as a stand-alone test, which is in agreement with the general recommendation to only employ serological assays for BTB in a parallel testing scheme with CMI-based diagnostics [19,43,72]. Similar results were obtained when the test performance of two rapid serological tests was evaluated in buffalo [73]. Although the specificity of the TB ELISA had a more acceptable range, the previously reported specificity of the assay in buffaloes of 100% could not be confirmed [19]. The test performance of the TB ELISA in cattle, although the Se is also variable, is much greater [13], suggesting that perhaps the assay is not optimal for use in buffaloes. In fact, the TB ELISA was developed for use in cattle specifically and makes use of an anti-bovine conjugate [24]. Cloete [74] recently demonstrated that the reactivity of an anti-bovine conjugate is significantly reduced in serum of African buffaloes as compared to cattle serum, which could explain the poor sensitivity of the assay shown in this study. As such, development of assays using either a species-specific or a broadly cross-reactive conjugate, such as protein A/G [74], would be recommended.

5. Conclusion

The impact of a long term BTB monitoring programme with removal of test positive animals in game reserves, such as in the HiP [40], was once more demonstrated by the significant difference in prevalence detected between the two parks and is key in order to reduce spill-over and spillback. This study has furthermore shown that the BTB prevalence affects the test performance of the TB ELISA in African buffaloes. This finding has direct implications for inclusion of this assay in testing strategies, since the sensitivities and specificities found in this study cannot be directly extrapolated to other epidemiological settings. As the test was found to have a high NPV in low prevalence herds, it could have merit if used to rule-out disease in known negative herds. However, the overall test performance of the TB ELISA in buffaloes was poor in this study, confirming it is not fit for purpose as a stand-alone test and suggesting it may not be suitable for use in this species. The findings of this study strongly point to the need for development of novel assays that can supplement existing assays for a more comprehensive testing scheme for BTB in African buffaloes. The potential development of a species-specific assay measuring HI and CMI simultaneously in a test platform suitable for the field (such as that described by Corstjens et al. [75]) would be highly valuable and warrants further investigation.

CRediT authorship contribution statement

Elisabeth M.D.L. van der Heijden: Conceptualization,

Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Funding acquisition. **David V. Cooper:** Investigation, Resources, Writing - review & editing, Supervision, Funding acquisition. **Victor P.M.G. Rutten:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **Anita L. Michel:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.cimid.2019.101369.

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