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Parallel testing increases detection of *Mycobacterium bovis*-infected African buffaloes (*Syncerus caffer*)

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ABSTRACT

The diagnosis of *Mycobacterium bovis* (*M. bovis*) infection in African buffaloes (*Syncerus caffer*) relies on detection of the cell-mediated immune response to *M. bovis* antigens using the single comparative intradermal tuberculin test (SCITT) or interferon gamma release assays (IGRAs). The aim of the present study was to determine whether parallel testing with the SCITT and an IGRA increases the number of *M. bovis*-infected buffaloes detected by these assays. Culture-confirmed animals ($n = 71$) tested during routine bovine tuberculosis (bTB) control programmes in Hluhluwe iMfolozi Park and Madikwe Game Reserve in South Africa, were used in this study. Results from 35 buffaloes tested using the SCITT and three Bovigam® IGRAs (cohort A) and 36 buffaloes tested using the SCITT, standard Bovigam® IGRA and Qiagen Cattletype IGRA (cohort B) were analysed. The parallel use of the SCITT with selected IGRAs was able to identify all animals in both cohorts. These findings are in agreement with cattle studies supporting the use of the SCITT and IGRAs in parallel to identify the greatest number of *M. bovis*-infected animals. The suggested parallel testing algorithm should be strategically applied to maximize detection of *M. bovis* infection in bTB-positive buffalo herds.

1. Introduction

Mycobacterium bovis (M. bovis) infection causes bovine tuberculosis (bTB), a disease affecting a diverse range of hosts including domestic pets, livestock, wildlife and humans (Michel et al., 2006). African buffaloes (*Syncerus caffer*) are sylvatic reservoirs of *M. bovis* and spill-over from buffaloes to wildlife, such as lions, and from buffaloes to livestock, such as cattle, has been demonstrated by molecular characterisation of mycobacterial strains (Hlokwe et al., 2014; Musoke et al., 2015). The first accounts of *M. bovis* infection in buffaloes in South Africa were documented in Hluhluwe iMfolozi Park (HiP) in 1986 (Jolles, 2004) and in Kruger National Park (KNP) in 1990 (Bengis et al., 1996). *M. bovis* infection has since been confirmed in numerous other wildlife species within these reserves, as well as more recently, in buffaloes in Madikwe Game Reserve (MGR) (Hlokwe et al., 2016) and privately-owned farms in South Africa.

Bovine tuberculosis is a chronic disease and progression is characteristically slow. Current methods to detect *M. bovis* infection rely on tests quantifying cell-mediated immune responses to mycobacterial antigens (Vordermeier et al., 2000). Such tests include the single

Abbreviations: ARC, Agricultural Research Council; bTB, bovine tuberculosis; CFP-10, culturefiltrate protein 10kD; ELISA, enzyme-linke dimmunosorbent assay; ESAT-6, early secretory antigen target 6kD; HiP, Hluhluwe iMfolozi Park; IFN-γ, interferon gamma; IGRA, inter feron gamma release assay; KNP, Kruger National Park; *M. bovis*, *Mycobacterium bovis*; MGR, Madikwe Game Reserve; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PPD_a, *Mycobacterium avium* purified protein derivative; PPD_b, *Mycobacterium bovis* purified protein derivative; QFT, QuantiFERON®-TB Gold; SCITT, single comparative intradermal tuberculin test; SFT, skin fold thickness

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comparative intradermal tuberculin test (SCITT) and interferon gamma (IFN-γ) release assays (IGRAs). The SCITT detects a delayed-type hypersensitivity response to intradermal injection of purified protein derivative (PPD), namely *Mycobacterium avium* PPD (PPD_a) and *M. bovis* PPD (PPD_b). The SCITT is currently the only test approved for the diagnosis of bTB in buffaloes in South Africa (Schiller et al., 2010).

The IGRA is an *in vitro* alternative to the SCITT and measures the release of IFN-γ in response to *M. bovis* antigens, using an enzyme-linked immunosorbent assay (ELISA) (Grobler et al., 2002). The standard Bovigam® IGRA is a commercially available test used for the diagnosis of bTB in cattle in which PPDs, like those used in the SCITT, are employed as stimulating antigens (de la Rua-Domenech et al., 2006). The use of a crude mix of mycobacterial antigens such as PPD has been shown to compromise IGRA specificity in buffaloes (van der Heijden et al., 2016) and as a result, a second generation of IGRAs have been developed using specific peptides as stimulating antigens to improve specificity (Parsons et al., 2011). The Bovigam® peptide IGRAs, Bovigam® PC-EC and Bovigam® PC-HP IGRA, use early secretory antigen target 6 kD (ESAT-6) in combination with culture filtrate protein 10 kD (CFP-10), and Rv3615 in combination with three additional proprietary mycobacterial antigens, respectively, as stimulating antigens (Goosen et al., 2014). The Qiagen Cattletype IGRA is a novel IGRA validated in buffaloes and makes use of the QuantiFERON®-TB Gold (QFT) tube stimulation system developed for humans containing ESAT-6 and CFP-10 stimulating antigens (Bernitz et al., 2018). The Bovigam® peptide IGRAs and Cattletype IGRA have demonstrated high test-specificity in buffaloes (Bernitz et al., 2018; Goosen et al., 2014)

This study aimed to determine if parallel testing of buffaloes in high bTB prevalence herds, using the SCITT and IGRAs, namely the standard Bovigam®, Bovigam® PC-EC, Bovigam® PC-HP and Cattletype IGRAs, increased the number of *M. bovis*-infected African buffaloes detected.

2. Materials and methods

2.1. Animals

Buffalo populations in HiP and MGR were screened for *M. bovis* infection as part of bTB testing programmes. Buffaloes were immobilised for the SCITT and blood collection, as previously described (Parsons et al., 2011). Buffaloes that tested positive on one or more tests were culled for post mortem examination. Lymph nodes, lungs and bTB-like lesions were examined and sampled for mycobacterial culture as previously described (Goosen et al., 2014) Culture-positive samples were genetically speciated using polymerase chain reaction (PCR) to confirm *M. bovis* infection (Warren et al., 2006). During 2015, 283 HiP buffaloes were tested using the SCITT and three Bovigam® IGRAs. Of these, 63 buffaloes were culled and 35 were culture-confirmed to be infected with *M. bovis* (cohort A) (Table 1). During 2016 and 2017, a total of 688 buffaloes from HiP and MGR were tested using the SCITT, standard Bovigam® and Cattletype IGRA. Of these, 94 were culled and 36 were culture-confirmed to be infected with *M. bovis* (cohort B) (Table 1). In total, 971 buffaloes were tested over a three-year period in HiP and MGR using the SCITT and a combination of IGRAs. Based on these results, 71 *M. bovis* culture-confirmed buffaloes were selected for inclusion in this study based on mycobacterial culture as the gold standard of *M. bovis* infection.

2.2. Bovigam® interferon gamma release assays (IGRAs)

In HiP, the standard Bovigam® IGRA was performed in a temporary field laboratory as previously described (Bernitz et al., 2018). Briefly, aliquots of heparinized whole blood were incubated in microcentrifuge tubes with i) phosphate buffered saline (PBS) ii) 2750 IU/ml PPD_a (Onderstepoort Biological Products, South Africa) iii) 3300 IU/ml PPD_b (Onderstepoort Biological Products) and iv) 5 μg/ml pokeweed mitogen (PWM) (Sigma-Aldrich, St. Louis, MO, USA). Samples were incubated at 37 °C for 20 h, the plasma fraction harvested, IFN-γ measured using the Bovigam® ELISA and results interpreted per manufacturer's instructions (Prionics AG, Schlieren-Zurich, Switzerland). The Bovigam® peptide IGRAs, i.e. Bovigam® PC-EC and Bovigam® PC-HP IGRAs, were performed as per the standard Bovigam® IGRA; however, 0.1 mg/ml PC-EC and 0.1 mg/ml PC-HP peptide cocktails (Prionics AG) were used as stimulating antigens, respectively. For MGR buffaloes, the standard Bovigam® IGRA was performed at the Tuberculosis Laboratory of the Agricultural Research Council (ARC)- Onderstepoort Veterinary Institute in Pretoria, South Africa with the following modifications to the standard Bovigam® protocol (van der Heijden et al., 2016). Aliquots of heparinized whole blood were incubated in 24-well cell-culture plates with i) 1000 IU/ml PPD_a (Prionics AG) ii) 600 IU/ml PPD_b (Prionics AG) and iii) 5 μg/ml PWM (Sigma-Aldrich). Aliquots of blood with no added antigen served as negative controls.

2.3. Qiagen Cattletype IGRA

The Cattletype IGRA was performed as previously described (Bernitz et al., 2018). Aliquots of heparinized whole blood were aseptically transferred to tubes of the QFT system (Qiagen, Venlo, Limburg, Netherlands) and then incubated at 37 °C for 20 h. The plasma fraction was harvested following centrifugation and the cattletype® ELISA (Qiagen) was performed per manufacturer's instructions.

2.4. Single comparative intradermal tuberculin test (SCITT)

The SCITT was performed on all buffaloes as previously described (Parsons et al., 2011). An area on either side of the buffalo's neck was shaved and the skin fold thickness (SFT) measured. The SCITT was performed by injecting 2500 IU PPD_a and 3000 IU PPD_b (Onderstepoort Biological Products) intradermally on either side of the buffalo's neck. After three days, buffaloes were immobilized again and the SFT at each injection site was measured. Buffaloes were considered test positive if the increase in SFT at the PPD_b site was 2 mm or greater than that at the PPD_a site.

Table 1
Tests performed in Hluhluwe iMfolozi Park in 2015, 2016 and 2017 and Madikwe Game Reserve in 2016 to detect *Mycobacterium bovis* infection in African buffaloes.

Population	Year tested	Cohort (n)	SCITT[a]	IGRA[b]			
				standard Bovigam®	Bovigam® PC-EC	Bovigam® PC-HP	Qiagen Cattletype
HiP[c]	2015	A (35)	√	√	√	√	–
	2016	B (36)	√	√	–	–	√
	2017		√	√	–	–	√
MGR[d]	2016		√	√	–	–	√

[a] single comparative intradermal tuberculin test.
[b] interferon gamma release assay.
[c] Hluhluwe iMfolozi Park.
[d] Madikwe Game Reserve.