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Field application of immunoassays for the detection of *Mycobacterium bovis* infection in the African buffalo (*Syncerus caffer*)

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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.

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 3000IU of PPD-B (purified protein derivative of *M. bovis*) (Prionics AG, Lelystad, the Netherlands) and 2500IU 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 vacutainer 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 μg/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 μg/ml) (internal positive control)