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IP-10: A potential biomarker for detection of *Mycobacterium bovis* infection in warthogs (*Phacochoerus africanus*)

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ABSTRACT

Bovine tuberculosis (bTB) is endemic in several areas of South Africa and has been reported in multiple species, including common warthogs (*Phacochoerus africanus*). Limited diagnostic tests and disease control programs exist for African wildlife. Thus, there is a need to develop techniques for bTB detection in species such as warthogs to assess their role in disease maintenance and spread in multi-host ecosystems. In this study, we obtained blood samples from warthogs in bTB endemic areas to investigate biomarkers for detection of *Mycobacterium bovis* infection. Warthog whole blood was incubated in QuantiFERON® TB Gold In-Tube tubes and pathogen specific release of interferon gamma (IFN-γ) and interferon gamma induced protein 10 (IP-10) was measured by a sandwich enzyme-linked immunosorbent assay. Although we were unable to measure IFN-γ, we could successfully measure IP-10. The IP-10 assay was able to distinguish between *M. bovis*-infected and *M. bovis*-culture negative warthogs, within bTB endemic areas, with an assay specific sensitivity of 68% and specificity of 84%. Of the 88 *M. bovis*-exposed warthogs screened, 42% were IP-10 test positive. These results indicate warthogs develop a measurable cell-mediated immune response after antigen stimulation of whole blood, which can distinguish between *M. bovis*-infected and *M. bovis*-culture negative animals. Thus, the IP-10 assay shows promise as an ante-mortem test to diagnose bTB in warthogs.

1. Introduction

Mycobacterium bovis causes bovine tuberculosis (bTB) in a wide variety of domestic and wildlife species in South Africa and is now endemic in some wildlife populations, with more than 17 species affected (Michel et al., 2006; Hlokwe et al., 2014). Currently there are limited bTB control programs for wildlife in South Africa to limit the risk of intra- and inter-species transmission (Hlokwe et al., 2014, 2016). This is a major concern as many parks and game farms are surrounded by cattle farmers or rural settlements.

Management strategies for bTB rely on accurate diagnostic tests for detection and surveillance. However, few diagnostic tools are available for testing most wildlife species, including common warthogs (*Phacochoerus africanus*). Warthogs can easily move between properties and may serve as potential maintenance hosts of *M. bovis*, similar to the role wild boar play in parts of Europe (de Lisle et al., 2002; Bengis et al., 2004; Naranjo et al., 2008). Therefore, techniques for detection of bTB in warthogs need to be developed to assess the role of this species in disease maintenance and spread in multi-host systems.

Serological assays have been developed that can distinguish between *M. bovis* culture-positive and culture-negative warthogs with a sensitivity (Se) and specificity (Sp) of 88% and 89% respectively (Roos et al., 2016). Using these tests, high seroprevalence (up to 63%) has been found in warthogs in bTB-endemic areas (Roos et al., 2018). However, serological assays for bTB in other species are reportedly less sensitive than those based on cell-mediated immune responses (CMI)

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(Maas et al., 2013).

In vitro tests commonly used to measure CMI to *M. bovis* include assays that detect antigen-specific production of interferon gamma (IFN-γ). However, reports of CMI assays for the detection of *M. bovis*-infected suids are limited. Pesciaroli et al. (2012) investigated the use of an IFN-γ assay in pigs, using bovine and avian purified protein derivative (PPD) to stimulate antigenic responses in whole blood (WB). Their results suggested that a swine IFN-γ assay could be a valuable component of a bTB surveillance program.

To improve the Sp of cytokine release assays, *M. bovis*-specific antigens, such as early secretory antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10), can be used in place of PPD. The QuantiFERON® TB Gold In-Tube (QFT) system utilizes peptides simulating these antigens for the diagnosis of *M. tuberculosis* infection in humans and has been successfully utilized to stimulate IFN-γ production in *M. bovis* infected wildlife (Parsons et al., 2011; Bernitz et al., 2018). The QFT stimulation platform has also been used to investigate other cytokine responses, such as interferon gamma induced protein 10 (IP-10), in *M. bovis* infection (Goosen et al., 2014a,b).

In addition to IFN-γ, IP-10 appears to be a promising biomarker for TB in humans and animals (Ruhwald et al., 2007; Goosen et al., 2015). As the name indicates, IP-10 is a chemokine induced by IFN-γ which plays a role in the delayed-type hypersensitivity response (Luster and Ravetch, 1987). Following antigen stimulation of WB, IP-10 is significantly more abundant than IFN-γ in human patients with active TB and may increase detection of individuals that have latent *M. tuberculosis* infection (Ruhwald et al., 2008; Mihret et al., 2013). Measurement of IP-10 also increased the Se of the QFT-based assay in African buffaloes compared to IFN-γ (Goosen et al., 2015).

Since IP-10 can be a sensitive biomarker for TB but has not been studied in wild suids, we hypothesized that evaluation of this chemokine may provide an additional tool for identifying warthogs with *M. bovis* infection. Therefore, the aim of this study was to develop and evaluate cytokine release assays measuring IFN-γ and IP-10 in QFT-stimulated WB for the detection of *M. bovis* infection in warthogs.

2. Materials and methods

2.1. Animals and sampling

Opportunistic samples were obtained from 88 warthogs within bTB endemic areas of South Africa. All warthogs were immobilized using a drug combination of zolazepam-tiletamine (Zoletil®; Virbac RSA, (Pty) Ltd, Centurion, South Africa) in combination with azaperone (Kyron Laboratories (Pty) Limited, Benrose, South Africa), medetomidine (Kyron), or azaperone, butorphanol (Kyron), medetomidine and ketamine (Kyron) see (Moon and Smith, 1996; Hewlett, 2017). Immobilizations and holding conditions complied with the South African National Parks Standard Operating Procedures for the Capture, Transportation and Maintenance in Holding Facilities of Wildlife. Blood was collected into Vacutainer® lithium heparin tubes (BD Biosciences, Franklin Lakes, New Jersey, USA). Post-mortem examinations were performed on 68 of these animals, as part of a disease surveillance program.

Head, cervical, thoracic, abdominal and peripheral lymph nodes were examined for the presence of gross lesions consistent with bTB. Lymph nodes were collected from warthogs for mycobacterial culture. In cases where no visible bTB-like lesions (NVL) were observed, lymph node samples were pooled per anatomical sample site, i.e. head and cervical (pool 1), thorax (pool 2), abdomen (pool 3) and peripheral (pool 4). Separate samples were collected from any tissue with visible bTB-like lesions (VL) and all tissues were frozen at −20 °C until processed for mycobacterial culture. Ethical approval was received from the Stellenbosch University Animal Care and Use committee (SU-ACUD15-00029).

2.2. Mycobacterial cultures and speciation

All tissues were processed for mycobacterial culture using the BACTEC™ MGIT™ 960 system (BD Biosciences) as previously described (Goosen et al., 2014a,b). All Ziehl-Neelsen stain-positive bacterial cultures were speciated using genetic region of difference analysis (Warren et al., 2006) and 16S DNA sequencing (Leclerc et al., 2000). Speciation results were used to define animals as *M. bovis*-infected, non-tuberculous mycobacteria (NTM)-infected, or culture-negative.

2.3. Whole blood stimulation and cytokine assays

All blood samples were processed within 8 h of collection. One ml of heparinised WB was added to each of three tubes: a QuantiFERON® (QFT) Nil tube (containing saline), a TB antigen tube (containing ESAT-6/CFP-10 and TB7.7 peptides) (Qiagen, Venlo, Netherlands) and a positive control tube, containing pokeweed mitogen (PWM) (Sigma-Aldrich, St. Louis, Missouri, USA) at a final concentration of 10 μg/ml. All samples were incubated for 24 h at 37 °C. After incubation, plasma was harvested following centrifugation at 800 *g* for 10 min. The plasma fraction was transferred to a 2 ml microcentrifuge tube and stored at −80 °C until analysed. Enzyme-linked immunosorbent assays (ELISA) were developed and optimised using commercially available antibodies (Ab) to the cytokines IFN-γ and IP-10, with recombinant IFN-γ and IP-10 at known concentrations as standards (Table 1). Assays were performed as previously described in detail (Clarke et al., 2017). The only exception was in the IFN-γ assay, where 12.5 μl of plasma and 37.5 μl of blocking buffer were used during the sample incubation step.

2.4. ELISA optimization

Plasma from five randomly selected warthogs, derived from PWM-stimulated blood (PWM-plasma) and blood incubated in QFT Nil tubes (Nil-plasma), was combined in a PWM-plasma pool and Nil-plasma pool, respectively. For the IFN-γ assay, each plasma pool was assayed using all combinations of capture antibody (cAb) and detection antibody (dAb) (Table 1). The optimal Ab combination was selected as the

Table 1
Commercial reagents screened for development of cytokine ELISAs.

Analyte	P[a]/M[b]	Manufacturer	Product information Kit or Catalogue no.
Bovine IP-10	P	Kingfisher Biotech Inc., St Paul, State, USA	cAb[c]: PB0385B−100 dAb[d]: PBB0393B−050 RP[e]: RP0079B−005
Bovine IFN-γ	P	Kingfisher Biotech Inc.,	cAb: PB0156B-100 dAb: PBB0267B-050 RP: RP0013B-005
Feline IFN-γ	P	Kingfisher Biotech Inc.,	cAb: PB0281F-100 dAb: PBB0283F-050 RP: RP0135F-005
Swine IFN-γ	P	Kingfisher Biotech Inc.,	cAb: PB0157S-100 dAb: PBB0269S-050 RP: RP0126S-005
Swine IFN-γ	M	Mabtech AB, Nacka Strand, Sweden	Kit no: 3130-1H-6
Bovine IFN-γ	M	Mabtech AB,	Kit no: 3115-1H-20
Feline IFN-γ (cAb) Bovine IFN-γ (dAb)	M	Serotech, Bio-Rad, Oxford, UK	cAb: MCA2140 dAb: MCA1783B
Human IFN-γ	M	BD Biosciences, San Jose, CA, USA	cAb: 551221 dAb: 554550

[a] Polyclonal.
[b] Monoclonal.
[c] Capture Antibody.
[d] Detection Antibody.
[e] Recombinant Protein.