# Detection and Characterization of Mycobacterial Infections Occurring in *Phacochoerus africanus* (Gmelin, 1788) (Common Warthog)

by

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Dissertation presented for the degree of Doctor of Philosophy (Molecular Biology) in the Faculty of Medicine and Health Sciences at Stellenbosch University

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# Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes 8 chapters and includes 3 original papers published in peer reviewed journals (Chapters 2-4) and 3 chapters of unpublished work (Chapters 5-7). The remaining 2 chapters include the general introduction (Chapter 1), general discussion and conclusion (Chapter 8). The development and writing of the papers (published and unpublished) were the principal responsibility of myself and for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

Date: December 2018

# Summary

*Mycobacterium bovis,* a member of the *Mycobacterium tuberculosis* complex and the cause of bovine tuberculosis (bTB), has an extensive host range that includes livestock and wildlife. While warthogs are considered spill-over hosts for bTB, they could potentially become reservoir hosts, if conditions are favourable, i.e. increased population densities. With limited knowledge on the infection status of warthogs in South Africa and their epidemiological significance for other species, it is imperative to have readily available diagnostic tests for warthogs.

Therefore, this study aimed to (i) establish reference cohorts of *M. bovis*-infected and uninfected warthogs; (ii) utilize these for the development and evaluation of diagnostic tools that can distinguish between infected and uninfected individuals*;* (iii) determine the seroprevalence of bTB in warthogs using the newly developed diagnostic tools; and (iv) characterize the genetic diversity of *M. bovis* isolates from warthogs.

Three serological assays, i.e. the indirect PPD ELISA, the TB ELISA-VK® and the DPP® VetTB Assay, could distinguish between *M. bovis*-infected and uninfected warthogs with high sensitivity (75-88%) and specificity (79-89%). The overall seroprevalence from four *M. bovis*-endemic locations was high, i.e. 38%. Furthermore, three tests measuring the cell-mediated immune responses of warthogs were developed. A cytokine release assay measuring interferon gamma induced protein 10 was able to distinguish between *M. bovis*-infected and uninfected warthog with a sensitivity of 68% and a specificity of 84%. The comparative intradermal tuberculin test classified 100% of culture-negative warthogs as test negative and 81% of culture-positive warthogs as test positive. Lastly, *CXCL9, 10, 11, IFNG*

and *TNFA* gene expression were significantly increased in whole blood from *M. bovis*-infected warthogs in response to antigen stimulation, with *CXCL10* showing the greatest mean fold increase. High genetic diversity of *M. bovis* isolates from warthogs was confirmed through spoligotyping and whole genome sequencing. Two distinct clades of *M. bovis* were identified by WGS, even though they shared the same spoligotype patterns.

This study has demonstrated that warthogs develop measurable and specific immune responses to *M. bovis* infection, which can be used to identify infected individuals ante-mortem. Furthermore, these tests will facilitate epidemiological studies of bTB in warthogs. With a high culture prevalence in warthogs from bTB endemic areas such as uMhkuze Nature Reserve and the Greater Kruger National Park, and high seroprevalence, warthogs seem to be highly susceptible to *M. bovis* infection. This suggests that warthogs may be an ideal sentinel species and strengthens the case that, under certain circumstances, they could be maintenance hosts. The genetic diversity of *M. bovis* isolates and the identification of two distinct clades challenges the current hypothesis that a single dominant strain circulates within a specific geographical location. Warthogs as a species should receive greater attention as potential disease maintenance hosts or as sentinels for bTB disease surveillance.

# Opsomming

*Mycobacterium bovis*, 'n lid van die *Mycobacterium tuberculosis* kompleks en die oorsaak van bees-tuberkulose (bTB), het 'n uitgebreide gashere stelsel wat strek vanaf vee tot-en-met wild. Terwyl vlakvarke as oorspoel gashere vir bTB beskou word, kan hulle potensieël instandhoudingsgashere wees, indien toestande gunstig is, d.w.s. wanneer daar ‘n hoë bevolkingsdigtheid is. Met ‘n beperkte kennis, ten opsigte van vlakvarke se infeksiestatus, in Suid-Afrika asook hul epidemiologiese betekenis vir ander spesies, is dit noodsaaklik om algemeen beskikbare diagnostiese toetse vir vlakvarke te hê wat besmette individue kan identifiseer.

Daarom het hierdie studie daarop gemik om (i) verwysingskohorte van *M. bovis*-besmette en onbesmette vlakvarke te bevestig; (ii) dié te gebruik vir die ontwikkeling en evaluering van diagnostiese instrumente wat tussen besmette en onbesmette individue kan onderskei; (iii) die seroprevalensie van bTB in vlakvarkke te bepaal, deur gebruik te maak van die nuut ontwikkelde diagnostiese instrumente; en (iv) die genetiese diversiteit van *M. bovis*-isolate van vlakvarke te karakteriseer.

Drie serologiese toetse, naamlik die indirekte PPD ELISA, die TB ELISA-VK® en die DPP® VetTB-assay, kan onderskei tussen *M. bovis*-besmette en onbesmette vlakvarke met hoë sensitiwiteit (75-88%) en spesifisiteit (79-89%). Die algehele seroprevalensie van vier *M. bovis*-endemiese gebiede was hoog, d.w.s. 38%. Nog drie toetse was ontwikkel wat die selbemiddelde immuunreaksie van vlakvarke gemeet het. 'n Sitokien-vrystellingstoets wat interferon-gamma-geïnduseerde proteïen 10 meet was in staat om te onderskei tussen *M. bovis*-besmette en onbesmette vlakvarke met 'n sensitiwiteit van 68% en 'n spesifisiteit van

84%. Die vergelykende intradermale tuberkulintoets het 100% van kultuur-negatiewe vlakvarke as toets-negatief geklassifiseer en 81% van kultuur-positiewe vlakvarke as toets positief geklassifiseer. Laastens, was die geen-uitdrukking van *CXCL9, 10, 11, IFNG* en *TNFA* beduidend hoër in die bloed van *M. bovis*-besmette vlakvarke, in reaksie op antigeenstimulasie, met *CXCL10* wat die grootste gemiddelde vouverhoging toon. Hoë genetiese diversiteit van *M. bovis*-isolate, vanuit vlakvarke, is bevestig deur middel van spoligotipering en heelgenoom volgorde bepaling. Twee verskillende stamme van *M. bovis* is deur heelgenoom volgorde bepaling geïdentifiseer, alhoewel hulle dieselfde spoeligotipepatrone gedeel het.

Hierdie studie het getoon dat vlakvarke ‘n meetbare en spesifieke immuunreaksie op *M. bovis*-infeksie ontwikkel, wat gebruik kan word om besmette individue ante-mortem (voor die dood) te identifiseer. Verder sal hierdie toetse epidemiologiese studies van bTB in vlakvarke fasiliteer. Met 'n hoë kultuurvoorkoms in vlakvarke afkomstig vanuit bTB-endemiese gebiede soos uMhkuze Natuur Reservaat en die Grooter Kruger Nationale Park, as ook die hoë seroprevalensie, lyk vlakvarke hoogs vatbaar vir *M. bovis*-infeksie. Dit dui daarop dat vlakvarke 'n ideale brandwag spesie kan wees en versterk die aanname dat hulle onder sekere omstandighede instandhoudingsgashere kan wees. Die genetiese diversiteit van *M. bovis*-isolate en die identifisering van twee duidelike stamme daag die huidige hipotese uit, wat voorstel dat 'n enkele dominante stam versprei word binne 'n bepaalde geografiese gebied. Vlakvarke as 'n spesie moet groter aandag geniet as ‘n moontlike instandhoudingsgasheer of as brandwag vir bTB.

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*Audere est Facere* – To do is to dare | Waag om te doen | Dare ukwenza

# Dedications

In memory of Christiaan Schotlz, on a well-played innings. To a man who dreamed as if he will live forever and lived as if he will die today. You are dearly missed my friend. Until we meet again, auf Wiedersehen!


I dedicate this work to God for the strength, courage and favour He has bestowed upon me.

**All the glory to You God!**

"Show me your ways, Lord, teach me your paths. Guide me in your truth and teach me, for You are God my Saviour, and my hope is in You all day long."

**Psalm 25:4-5**

"For our light and momentary troubles are achieving for us an eternal glory that far outweighs them all. So we fix our eyes not on what is seen, but on what is unseen, since what is seen is temporary, but what is unseen is eternal."

**2 Corinthians 4:17-18**

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# List of abbreviations

| | | |
|---|---|---|
| Ab | - | Antibody |
| AUC | - | Area under the curve |
| Avg. | - | Average |
| B2M | - | Beta-2 microglobulin |
| BB | - | Blocking buffer |
| BD | - | Becton Dickinson |
| BIC | - | Bayesian information criterion |
| BLAST | - | Basic Local Alignment Search Tool |
| bTB | - | Bovine tuberculosis |
| BWA | - | Burrows-Wheeler Aligner |
| cAb | - | Capture antibody |
| cDNA | - | Complementary deoxyribonucleic acid |
| CA | - | California |
| CFP-10 | - | 10 kDa culture filtrate antigen |
| CI | - | Confidence interval |
| CITT | - | Comparative intradermal tuberculin test |
| CMI | - | Cell mediated immune response/s |
| CN | - | Culture negative |
| CP | - | Culture positive |
| CXCL11 | - | Chemokine (C-X-C motif) ligand 11 (Gene: *CXCL11*) |
| Cq | - | Quantification cycle |
| dAb | - | Detection antibody |
| DE | - | Delaware |
| DM | - | Derivative melt curve |

| | | |
|---|---|---|
| DNA | - | Deoxyribonucleic acid |
| DPP® TB | - | Dual-path platform technology for tuberculosis |
| EI | - | ELISA Index |
| ELISA | - | Eenzyme-linked immunosorbent assay |
| ESAT6 | - | 6 kDa early secretory antigenic target |
| GATK | - | Genome Analysis Tool Kit |
| gDNA | - | Genomic deoxyribonucleic acid |
| GEA | - | Gene expression assay |
| GKNP | - | Greater Kruger National Park |
| GTR | - | General time reversal |
| h | - | Hours |
| H3F3A | - | Histone H3.3 |
| IA | - | Iowa |
| INF-γ | - | Interferon gamma (Gene: *INFG*) |
| IP-10 | - | Interferon gamma induced protein 10 |
| | | Chemokine (C-X-C motif) ligand 10 (Gene: *CXCL10*) |
| IGRA | - | Interferon gamma release assay |
| ITT | - | Intradermal tuberculin test |
| IQR | - | Inter quartile range |
| IU | - | International units |
| kDa | - | Kilodalton |
| KNP | - | Kruger National Park |
| KZN | - | KwaZulu-Natal |
| LDHA | - | Lactate dehydrogenase A |
| M | - | Monoclonal |

| | | |
|---|---|---|
| Mdn | - | Median |
| MGIT | - | Mycobacterial growth indicator tubes |
| MIG | - | Chemokine (C-X-C motif) ligand 9 (Gene: *CXCL9*) |
| Min | - | Minutes |
| MIRU-VNTR | - | Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats |
| MK | - | uMhkuze Nature Reserve |
| ML | - | Maximum likelihood |
| mm | - | Millimetre |
| MO | - | Missouri |
| MP | - | Marloth Park |
| MPB70 | - | Major secreted immunogenic protein 70 (Gene: *Rv2875*) |
| MPB83 | - | Major secreted immunogenic protein 83 (Gene: *Rv2873*) |
| mRNA | - | Messenger ribonucleic acid |
| MTBC | - | *Mycobacterium tuberculosis* complex |
| µg | - | Microgram |
| µl | - | Microliter |
| µM | - | Micromolar |
| ml | - | Millilitre |
| MZ | - | uMhkuze Nature Reserve |
| N | - | Negative |
| NCBI | - | National Center for Biotechnology Information |
| ND | - | Not done |
| ng | - | Nanogram |
| NJ | - | New Jersey |

| | | |
|---|---|---|
| NMC | - | Negative mycobacterial culture |
| nm | - | Nanometre |
| NRF | - | National Research Foundation |
| NTM | - | Non-tuberculous mycobacteria |
| NVL | - | No visible lesion |
| NY | - | New York |
| OD | - | Optical density |
| OIE | - | World Organisation for Animal Health (Office International des Epizooties) |
| OR | - | Odds ratio |
| P | - | Positive or Polyclonal |
| PBS | - | Phosphate buffered saline |
| PC | - | Positive control |
| PCR | - | Polymerase chain reaction |
| pg | - | Picogram |
| PPD | - | Purified protein derivative |
| $PPD_a$ | - | Avian purified protein derivative |
| $PPD_b$ | - | Bovine purified protein derivative |
| PPIA | - | Peptidylprolyl isomerase A |
| PWM | - | Pokeweed mitogen |
| QFT | - | QuantiFERON® |
| qPCR | - | Quantitative polymerase chain reaction |
| RaxML | - | Randomized Axelerated Maximum Likelihood |
| RD | - | Regions of difference |
| rDNA | - | Ribosomal deoxyribonuclease acid |

| | | |
|---|---|---|
| RFLP | - | Restriction fragment length polymorphism |
| RLU | - | Relative light units |
| ROC | - | Receiver operating characteristics curve |
| RNA | - | Ribonucleic acid |
| RP | - | Recombinant protein |
| RSA | - | Republic of South Africa |
| RT | - | Room temperature or Reverse transcription |
| RT-qPCR | - | Reverse transcription-quantitative polymerase chain reaction |
| s | - | Seconds |
| SA | - | South Africa or Satara |
| SANParks | - | South African National Parks |
| SARChI | - | South African Research Chair Initiative |
| SD | - | Standard deviation |
| Se | - | Sensitivity |
| SFT | - | Skin fold thickness |
| SITT | - | Single intradermal tuberculin test |
| SK | - | Skukuza |
| SNP | - | Single nucleotide polymorphisms |
| Sp | - | Specificity |
| SU | - | Stellenbosch University |
| TB | - | Tuberculosis |
| TMB | - | 3,3',5,5'-Tetramethylbenzidine |
| TNF-α | - | Tumour necrosis factor alpha (Gene: *TNFA*) |
| TX | - | Texas |
| USA | - | United States of America |

| | | |
|---|---|---|
| USDA | - | United States Department of Agriculture |
| VK | - | Vacunek |
| VL | - | Visible lesion |
| VNTR | - | Variable number tandem repeat typing |
| WB | - | Whole blood |
| WGS | - | Whole Genome Sequencing |
| YWHAZ | - | Tyrosine-3-mono-oxygenase/tryptophan-5-monooxygenase activation protein zeta |
| ZN | - | Ziehl–Neelsen |

# Chapter 1

## General Introduction:

## The common warthog as a host of *Mycobacterium bovis*

**Eduard O. Roos**

This introductory chapter aims to give a literature summary of the host, pathogen and the diagnosis of the disease. Furthermore, it will highlight the justification for a bovine tuberculosis study in warthogs as well as setting the research question, study aims, objectives, and describing the originality of the study.

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**My contribution to this chapter:** Review of literature
Planning of project
Writing of chapter

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**Common Warthog (*Phacochoerus africanus*)**

Warthogs are a wild suid consisting of two species, the desert warthog (sub-species: *Phacochoerus aethiopicus*, extinct and *P. a. delamerei*) and the common warthog (sub-species: *P. africanus africanus*, *P. a. aeliani*, *P. a. massaicus* and *P. a. sundevallii*) (d'Huart and Grubb 2003; Grubb and D'Huart 2010). The common warthog is the most abundant and widespread wild suid species in Africa and occurs across most of the continent (Fig. 1.1) (Muwanika et al. 2003). The desert warthog is only found in the horn of Africa (Fig. 1.2) (d'Huart and Grubb 2003). Due to habitat fragmentation and clearance for agricultural land, warthogs have become restricted to protected areas (Muwanika et al. 2003). They have also been reintroduced to various provincial and national game reserves in South Africa as well as private game farms, as they are considered charismatic animals (Swanepoel 2016).

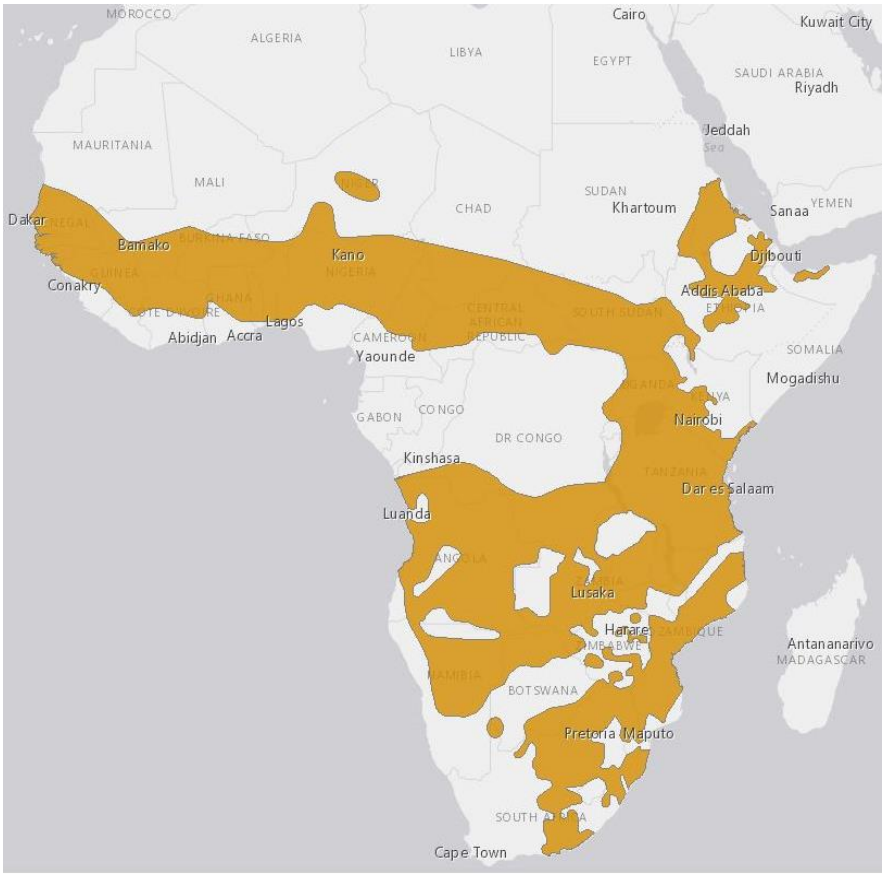


**Figure 1.1** Map indicating the continental distribution of all sub-species of the common warthog (de Jong et al. 2016).

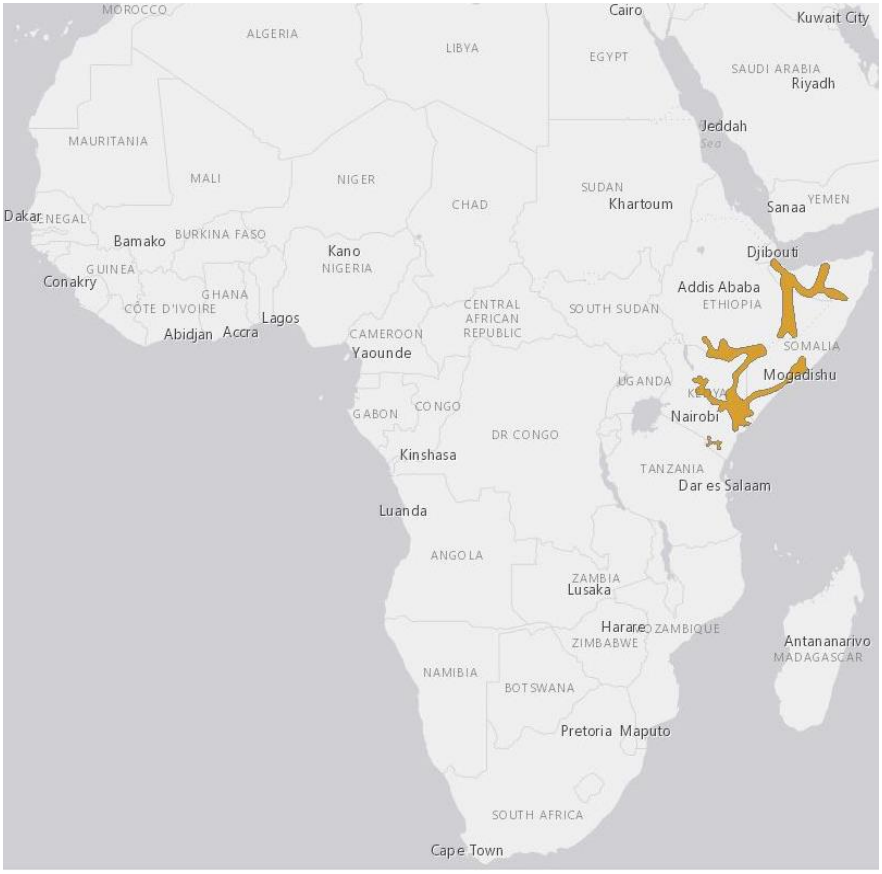


**Figure 1.2** Map indicating the continental distribution of all sub-species of the desert warthog (de Jong et al. 2016).

Similar to other suids, warthogs are not easily confined by most wire fencing used on farms and reserves in South Africa and thus many became free-roaming in their introduced habitats (Swanepoel 2016). Moreover, warthogs are predominantly grazers and feed on a wide variety of grasses, although they also consume other food items such as bulbs, tubers and fruit (Mason 1982, 1990). They have also occasionally been observed feeding on carcasses in Kruger National Park (pers. comm. Dr Peter Buss, 2016). Warthogs are generally seen as a pest species by the agricultural sector, as they can decimate crops, grass cover, destroy fences and their foraging behaviour may impact vegetation and landscape structures negatively (Nyafu 2009). Furthermore, these wild suids are carriers of various pathogens including African swine fever virus, Foot-and-Mouth Disease virus, Trypanosomes, and

*Mycobacterium bovis*, which may infect other animals or humans (Mason 1982; Miller et al. 2016).

Being predominantly grazers, warthogs are very susceptible to drought, often being the first species to suffer high mortalities during these periods (Mason 1990). Juveniles are the most susceptible to environmental stress and predation (Mason 1990). Although at times they experience high mortality that is associated with the local conditions, their high reproductive success rate ensures that their numbers bounce back rapidly as soon as the environment becomes favourable (Mason 1982, 1990). Litter sizes average between 2-4 piglets (Cumming 1975; Boshe 1981; Somers and Penzhorn 1992).

By understanding the biology and life history of warthogs, it becomes clearer how they can potentially transmit various diseases. Warthogs form strong social structures, which increase the chance of disease transmission between individuals. They form either bachelor groups that range between 2-4 adults and sub-adult males or family groups ranging from 2-9, consisting of individuals from all age groups (Mason 1990). Their philopatric behaviour result in warthogs not dispersing far from their maternal home ranges (1.44-6.97 $km^2$), possibly limited by their burrowing behaviour (White and Cameron 2009, 2011). Burrowing also increases chances of close contact with infected individuals. Warthogs exhibit helping behaviour in rearing their young and are thus facultative cooperative breeders (White and Cameron 2009, 2011). This may lead to potential disease transmission from a helper to juveniles or *vice versa*. Furthermore, warthogs are known to aggregate around watering holes where they frequently wallow for various beneficial reasons (e.g. thermoregulation) (Bracke 2011; Swanepoel 2016). However, wallowing behaviour and foraging on supplemental feed

have been reported as routes of *Mycobacterium bovis* transmission in wild boar (Payne et al. 2017).

***Mycobacterium bovis***

The *Mycobacterium tuberculosis* complex (MTBC) is a group of mycobacteria that originated in humans, roughly 40 000 years ago in East Africa, and over time has spread to animals (Wirth et al. 2008). These members are all closely related with high genetic similarity (Brosch et al. 2002; Smith et al. 2006). For example, the genome of *M. bovis* is 4.34 Mb, consisting of 3951 genes, with >99.95% homology with *M. tuberculosis* at the nucleotide level (Garnier et al. 2003). Members of the complex are pathogenic, and many of the members are associated with specific host species (i.e. *M. suricattae* in meerkats and *M. mungi* in banded mongoose) while others have adapted to survive and thrive in a wide range of host species (i.e. *M. tuberculosis* and *M. bovis* in humans, livestock and wildlife) (Smith et al. 2006; Clarke et al. 2016).

*M. bovis* can be genetically differentiated from the other MTBC using spoligotyping and region of difference (RD) analysis (Warren et al. 2006; Smith et al. 2006). Spoligotyping is commonly used to differentiate specific strains of *M. bovis* by identifying the polymorphism of spacers that occur within the direct repeat regions of the *M. bovis* genome (Smith et al. 2006). The standard spoligotype protocol uses a subset of 43 spacer units, selected from *M. tuberculosis* and *M. bovis*, that allows differentiation between MTBC (Smith et al. 2006). The RD analysis is also often used as a screening tool for speciating MTBC. Thus, RD analysis investigates the presence and absence of various RDs, which form species specific patterns when visualised on a gel (Warren et al. 2006; Smith et al. 2006). For *M. bovis*, the presence

of RD1 and absence of RD 4, 9, 12, distinguishes it from the other MTBCs (Fig. 1.3) (Warren et al. 2006).

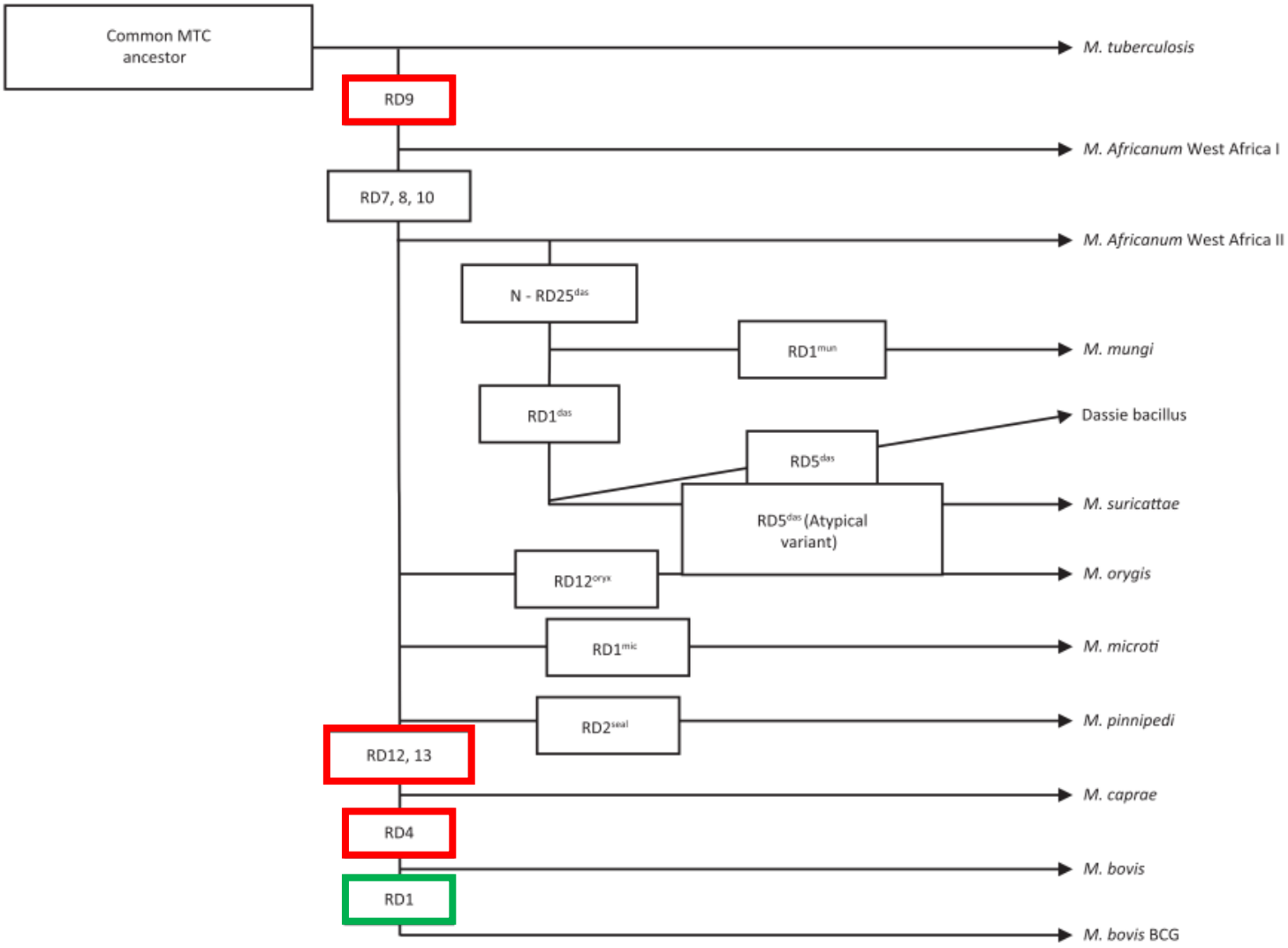


**Figure 1.3** Phylogenetic relationship of the MTBC. The region of difference (RD) in each block indicates the deletion (absence) of the specific RD from the genome. Of interest to M. bovis is RD 4, 9 and 12 which are deleted (red) and RD 1 which remains present (green) in the genome. Figure adapted from Clarke et al. (2016).

Another method to differentiate species is through whole genome sequencing (WGS) of the bacteria (Dippenaar et al. 2015, 2017). This method allows differentiation between bacterial strains by comparing the number of single nucleotide polymorphisms, large and small

deletions, or insertions (Dippenaar et al. 2015). WGS facilitates genetic characterisation of different strains as well as providing information on the molecular epidemiology of infection (Biek et al. 2012; Black et al. 2015; Crispell et al. 2017). Investigating the molecular epidemiological of *M. bovis*, the genetic diversity is often found to be geographically localized and has been described in both livestock and wildlife (Smith et al. 2006; Dippenaar et al. 2017; Machado et al. 2018). Therefore, it seems once *M. bovis* has entered an ecosystem, a single dominant strain is usually found and spreads through the geographical location (Kukielka et al. 2013; Dippenaar et al. 2017). Thus, WGS can help to determine the source and spread of the infection.

The wide host range of *M. bovis* is an important factor in the epidemiology of bovine tuberculosis (Marcotty et al. 2009; Miller and Olea-Popelka 2013; De Garine-Wichatitsky et al. 2013; Musoke et al. 2015; Cowie et al. 2016). More than 60 mammal species have been reported with bovine tuberculosis (bTB) worldwide (de Lisle et al. 2002; Good and Duignan 2011). While *M. bovis* is mostly isolated from cattle, other domestic species that may be infected include pigs, sheep, dogs, and cats, while wildlife species such as wild boar, possums, badgers, and African buffalo have become maintenance hosts in their respective ecosystems (O'Reilly and Daborn 1995; Renwick et al. 2007). Inter- and intraspecies transmission of *M. bovis* has been documented on every continent, except Antarctica (O'Brien et al. 2002; Corner 2006; Gortázar et al. 2007; Awah-Ndukum et al. 2013; Mol et al. 2016). Examples include the well-studied badger versus cattle case in the United Kingdom and free-roaming African buffalo in a transfrontier conservation area in southern Africa (Sweeney et al. 2007; Böhm et al. 2009; Biek et al. 2012; Caron et al. 2016). Consequently, the presence of wildlife reservoirs compounds the difficulty of controlling and eradicating the disease (Palmer 2007; Good and Duignan 2011).

Transmission of *M. bovis* is primarily via the respiratory route, for example in cattle and African buffaloes (O'Reilly and Daborn 1995). However, in many species, ingestion is the predominant route of infection, as in wild boar and feral pigs (Renwick et al. 2007; Santos et al. 2009; Vieira-Pinto et al. 2011). This is likely related to scavenging behaviour in suids (Vieira-Pinto et al. 2011). Therefore, warthogs could be infected when scavenging on infected carcasses or trash; also feeding or drinking at aggregation points such as watering holes (Barasona et al. 2014, 2017).

*M. bovis* is a hardy bacteria that can survive up to 20 days in corn or hay fed to animals during the northern hemisphere spring season, and in soil for up to 88 days (Fine et al. 2011). However, there is a decline in bacterial survival under summer conditions, for example, to only 11 days in soil (Fine et al. 2011). Even under South African climatic conditions, the bacteria can be viable in carcasses of infected animals for up to 42 days and in faeces, up to 28 days during winter; and 21 days in carcasses and 8 days in faeces in summer (Tanner and Michel 1999). Therefore, *M. bovis* survival in the environment, especially at aggregation points, can lead to indirect transmission (Barasona et al. 2017; Payne et al. 2017).

Being a slow-growing intracellular pathogenic mycobacterium, *M. bovis* can cause severe pathological damage when granulomas reach a type IV classification (van Helden et al. 2009; Michel et al. 2010; Domingo et al. 2014). These types of lesions are often confluent, having a caseous necrotic mineralised appearance, are extensive, and have well-developed fibrous encapsulations (Domingo et al. 2014). However, pathological changes can vary between species (Domingo et al. 2014). There are also significant pathological differences between *M. bovis* strains as each strain has a different virulence factor (Waters et al. 2014). Thus, the

pathology observed during *M. bovis*-infection appears to be a result of differences in the host's immune response and bacterial virulence.

The presence of *M. bovis* has a significant impact on animal health, production and trade worldwide (Michel et al. 2010). It is reported that the agricultural sector loses US$3-4 billion per year due to bTB, with more than 50 million cattle infected by 1995 (Garnier et al. 2003; Palmer et al. 2012; Maggioli et al. 2015). Between 2015 and 2017, more than 170 countries reported the presence of bTB in livestock or wildlife, highlighting the vast global distribution of *M. bovis* (Fig 1.4 and 1.5). In addition to the impact on animal health, the global burden of bTB as a zoonotic disease is high with 147,000 new cases and 12,500 deaths in 2016, although this estimate is imprecise due to a lack of surveillance for bTB in humans and the inability of the commonly used sputum microscopy diagnostic test to differentiate between *M. tuberculosis* and *M. bovis* (World Health Organization et al. 2017).

Few surveillance programs exist across the African continent for the control or eradication of bTB, although the disease is wide spread (Fig 1.4 and 1.5) (Renwick et al. 2007; De Garine-Wichatitsky et al. 2013). Another concern is that many countries in Africa do not report the bTB status of their wildlife (Fig 1.4 and 1.5) (De Garine-Wichatitsky et al. 2013). This can lead to increased risk to various conservation efforts, especially when endangered species need to be moved to maintain genetic diversity, but originate from *M. bovis* endemic areas, which could potentially introduce disease into a bTB-free region (Milliken et al. 2009; De Garine-Wichatitsky et al. 2013; Miller et al. 2017).

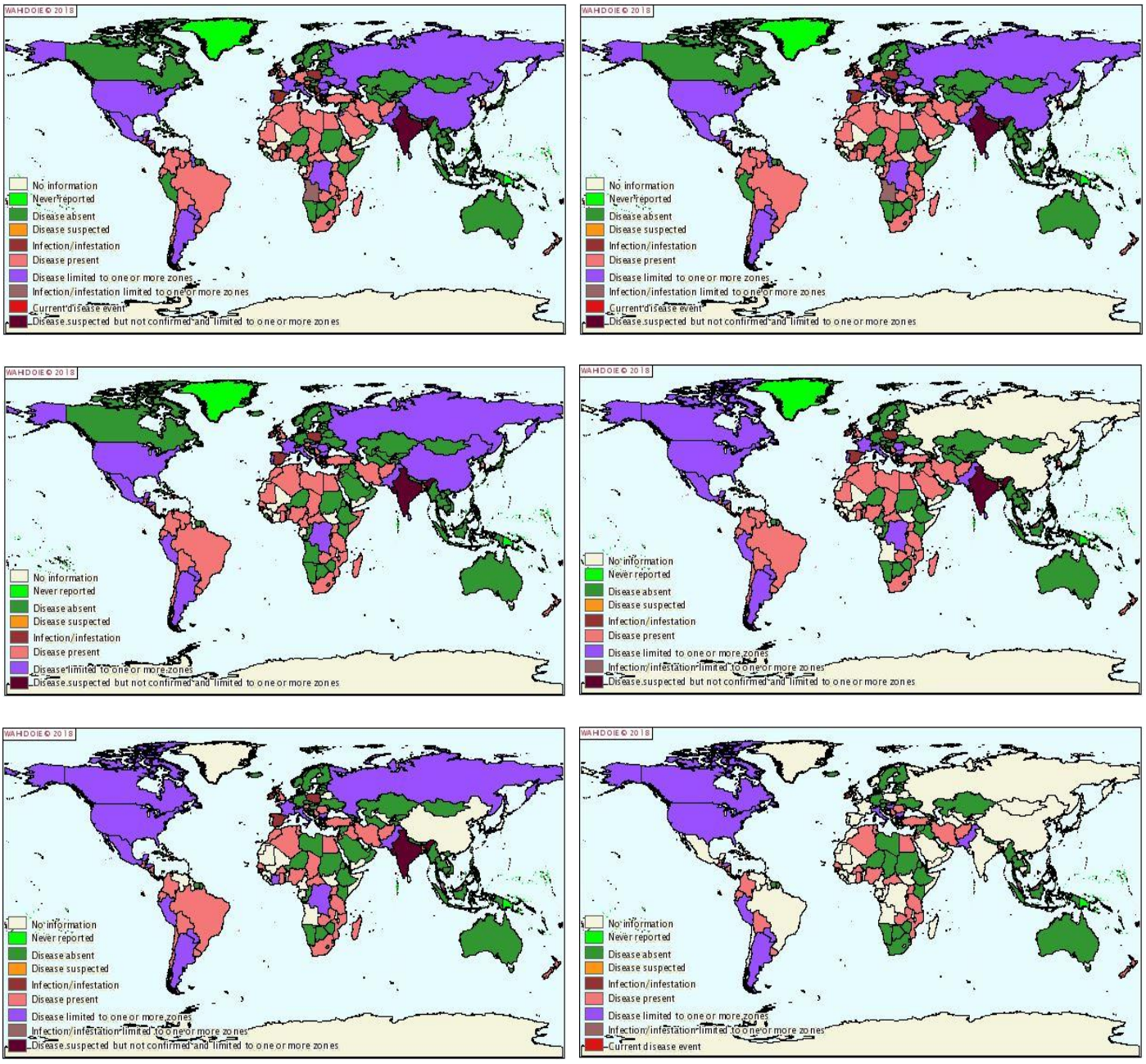


**Figure 1.4** Reports of bTB in livestock sent to the OIE for six-month periods (Jan-Jun, Jul-Dec) from 2015 to 2017. Figures are left to right Jan-Jun and Jul-Dec and top to bottom 2015, 2016 and 2017. White: No information; Lime green: Never reported; Dark green: Disease absent; Orange: Disease suspected; Light red: Infection/infestation; Pink: Disease present; Purple: Disease limited to one or more zones; Grey: Infection/infestation limited to one or more zones; Maroon: Disease suspected but not confirmed and limited to one or more zones. From: http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap

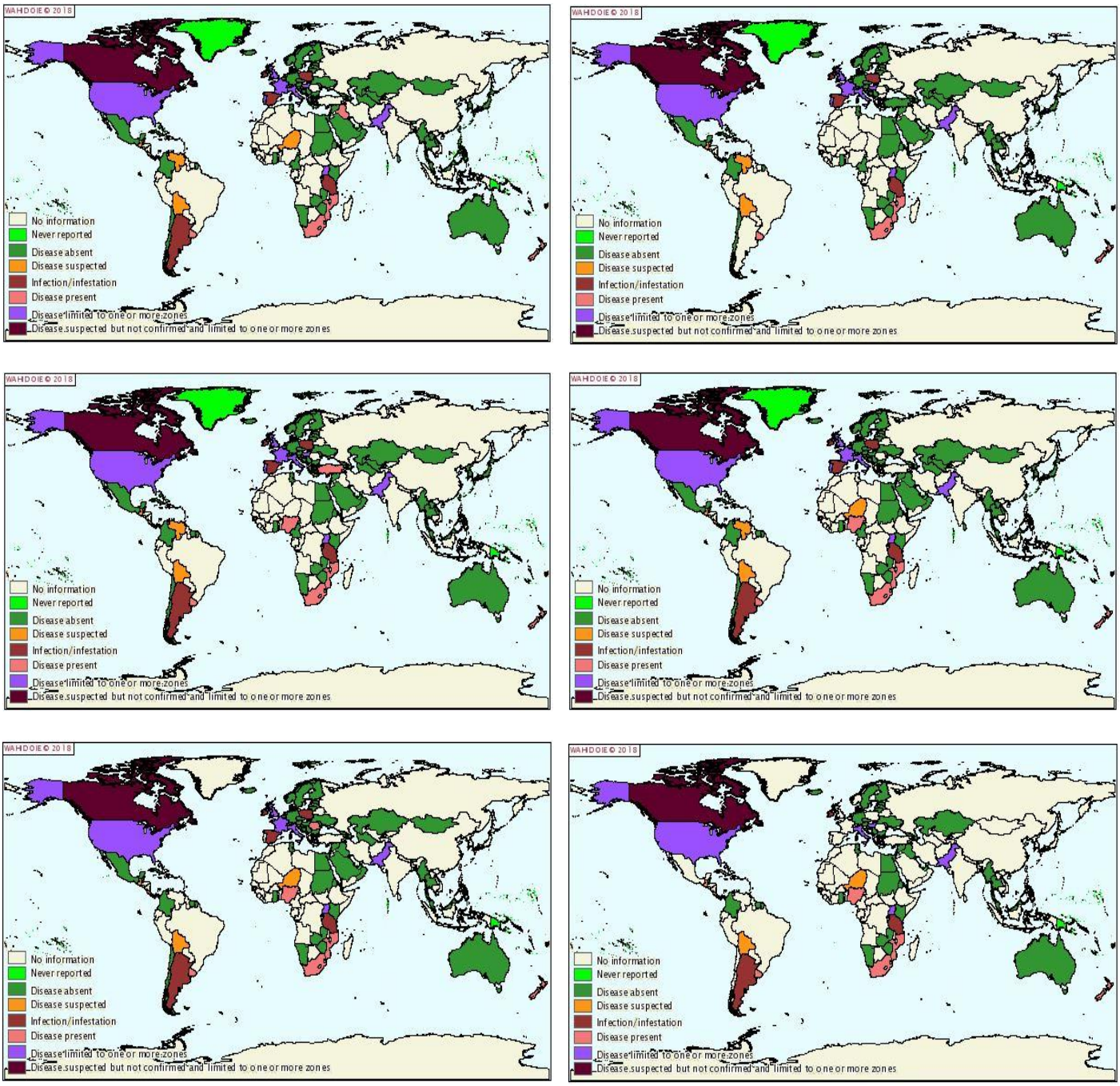


**Figure 1.5** Reports of bTB in wildlife sent to the OIE for six-month periods (Jan-Jun, Jul-Dec) from 2015 to 2017. Figures are left to right Jan-Jun and Jul-Dec and top to bottom 2015, 2016 and 2017. White: No information; Lime green: Never reported; Dark green: Disease absent; Orange: Disease suspected; Light red: Infection/infestation; Pink: Disease present; Purple: Disease limited to one or more zones; Grey: Infection/infestation limited to one or more zones; Maroon: Disease suspected but not confirmed and limited to one or more zones. From: http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation /Diseasedistributionmap

**Wild boar, feral swine and bTB**

In the Mediterranean ecosystem, wild boar play an important role in maintaining *M. bovis* and transmitting it to livestock and wildlife that share habitats (Naranjo et al. 2008; Kukielka et al. 2013; Barasona et al. 2014). Affected individuals may shed mycobacterial bacilli into the environment, thus serving as a source of infection for other species, and potentially humans (Naranjo et al. 2008; Meng et al. 2009; Richomme et al. 2013). Since swine can easily cross artificial barriers and move between properties, the presence of infected animals may be an under-recognized threat in the maintenance and geographic spread of bTB. Furthermore, studies have shown how useful wild boar and feral pigs can be for disease surveillance when used as sentinels (Nugent et al. 2002; Naranjo et al. 2008; Bailey et al. 2013).

Ideal sentinels for bTB should have a high susceptibility to *M. bovis*-infection, be relatively resistant to developing disease or disease-related mortality and present a low risk of infection to individuals collecting samples during necropsy or ante-mortem sampling (Nugent et al. 2002). Feral suids seem to fit this description as they are highly susceptible, have few clinical signs, and develop encapsulated lesions in their lymph nodes (limiting the risk to individuals collecting samples) (Nugent et al. 2002; Naranjo et al. 2008). Additionally, a good sentinel species should be abundant and cover a wide range as well as be easy to sample (Nugent et al. 2002).

Disease surveillance testing of wild boar and feral pigs has relied heavily on post-mortem examination and serological assays (Aurtenetxe et al. 2008; Boadella et al. 2011; Cardoso-Toset et al. 2015; Nugent et al. 2015). Neither of these two approaches are 100% sensitive (serological assays are also not 100% specific) when used as bTB diagnostic tests in wild

boar and feral pigs. Few studies have reported an alternative method of bTB diagnosis for these species. Although a significant amount of research has gone into the development of cell-mediated immune (CMI) response assays in other species such as cattle and buffalo, a handful have investigated its utility in wild boar and feral pigs (Gormley et al. 2006; Pesciaroli et al. 2012; Goosen et al. 2014a; Sinclair et al. 2016; Bernitz et al. 2018).

**Diagnosis of bTB**

The diagnosis of bTB in animals can be done using post- and ante-mortem techniques. Unfortunately, clinical diagnosis of bTB is often difficult and by the time clinical signs are observed, the disease is often advanced (de Lisle et al. 2002; Ramos et al. 2015). Although bTB diagnostic assays are available for livestock species, few have been modified for use in wildlife species (Michel et al. 2006; Broughan et al. 2013). Most of the bTB diagnostic assays have yet to be optimised and validated for non-bovid species (Cousins and Florisson 2005; Chambers 2009; Broughan et al. 2013). Difficulties in validating tests for wild species, including access to samples from known infected and uninfected animals in sufficient numbers, are a major obstacle for diagnosing bTB (Cousins and Florisson 2005; Chambers 2009).

Post-mortem techniques are commonly used in animals, with the gold standard being detection of *M. bovis* through mycobacterial culturing of lymph nodes, along with gross pathological and histopathological changes consistent with bTB (including acid-fast staining) as secondary tests (de Lisle et al. 2002; Maas et al. 2013; Ramos et al. 2015; Mol et al. 2016; Maciel et al. 2018). However, these techniques may have suboptimal sensitivity and culling of individuals may not be ideal for disease surveillance in different populations (de Lisle et al. 2002; Maas et al. 2013). The post-mortem tests are often expensive and time-consuming,

as well as lacking a sufficient combination of test sensitivity and specificity (de Lisle et al. 2002). Their major advantage is that samples do not need to be examined immediately, especially once frozen or fixed in formalin or on a slide.

Ante-mortem techniques are available, with most based on detecting host immune responses (Chambers 2013; Maas et al. 2013). However, there are some ante-mortem techniques which permit direct detection of mycobacterial organisms by microscopy, culture or PCR in faecal, oropharyngeal, nasal or bronchoalveolar samples (Michel et al. 2010, 2017; Broughan et al. 2013; Miller et al. 2015). However, tests that rely on detection of bacilli in ante-mortem samples are often not as sensitive as those that rely on the *M. bovis*-specific host immune response since culture results may be affected by intermittent shedding into secretions and bacterial load (Michel et al. 2010; Ghielmetti et al. 2017).

Indirect ante-mortem tests for bTB are dependent on the host's anamnestic response to specific antigens of *M. bovis* (Pai et al. 2004; Maggioli et al. 2015). The tuberculin skin test is a widely used *in vivo* test, although it has not been validated in many species. Disadvantages of this test include the need to handle an animal twice and the interpretation of the test is subject to operator bias (Montali and Hirschel 1990; de la Rua-Domenech et al. 2006; Maas et al. 2013). Other CMI response assays, such as interferon-gamma (IFN-γ) release assays and cytokine gene expression assays, have been shown to be more sensitive but have not been evaluated in many non-bovid species (Chambers 2009). However, these tests are limited as they can be logistically challenging to perform on large numbers of samples during surveillance and may have decreased sensitivity in animals with advanced disease (de la Rua-Domenech et al. 2006).

Serological assays on the other hand have shown promise as potential surveillance tools in some species, such as wild boar, cervids and elephants (de la Rua-Domenech et al. 2006; Aurtenetxe et al. 2008; Chambers 2009; Maas et al. 2013). These humoral assays for bTB detection generally focus on the use of $PPD_b$ as an array of antigens to capture IgG antibodies that are detected by conjugated Protein-G (Ritacco et al. 1990). However, tests based on humoral responses are not as sensitive as those based on the CMI response in many species, specifically during early stages of infection (de la Rua-Domenech et al. 2006). Cell-mediated immune responses, either *in vivo* or *in vitro*, are measured by the delayed type hypersensitivity reaction at the injection site, or production of cytokines and chemokines in stimulated WB to specific peptide/antigens (Monaghan et al. 1994; Vordermeier et al. 2001). Therefore, bTB diagnostic test development requires an understanding of specific immune responses in each species.

During the development and validation of any bTB diagnostic test, it is important to factor in the presence of non-tuberculous mycobacteria (NTM) and the test's ability to distinguish between *M. bovis* and NTM infection (Andersen et al. 2000; Pai et al. 2004). Current CMI response assays often use antigens that are coded for by genes present in the RD1 region, which is found in the MTBC and absent in *M. bovis* BCG and most other NTMs. These include, early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) which make these tests more specific to MTBC (Pai et al. 2004). In contrast, purified protein derivative (PPD) is a crude mixture of antigens and is often used in serological assays, although the presence of shared antigens with NTMs decreases the assay's specificity (Hanna et al. 1989; Lilenbaum et al. 1999; Pai et al. 2004; Aurtenetxe et al. 2008). Therefore, studies have aimed to improve the specificity of these serological assays by utilizing antigens such as recombinant ESAT-6/CFP-10, MPB70 and MPB83 that are specific for the MTBC and

reduce the cross reactivity with NTMs, compared to PPD (Harboe et al. 1990, 1996; Lyashchenko et al. 2000, 2018; Wiker 2009).

Each type of assay has advantages and limitations. Factors which influence test performance and selection include the stage of infection, presence of disease, host's immune status, exposure to environmental mycobacteria, and variability in immune responses between species. Therefore, it is important to assess the assay performance in a specific species and environments in which these assays will be used to optimize detection of infected individuals.

**Justification for bTB study in warthogs**

Warthogs are a close relative of wild boar and feral pigs and share behavioural and ecological niches within their respective ecosystems (Fig. 1.6) (Chen et al. 2007). They have similar litter sizes of 2-8 piglets per litter (Boshe 1981; White and Cameron 2009; Nugent et al. 2015). All pigs show typical wallowing behaviour, that could lead to a potential route of *M. bovis* transmission in these species (VerCauteren et al. 2007; Bracke 2011). Wallowing behaviour and foraging on supplemental feed have been reported as routes of *Mycobacterium bovis* transmission in wild boar (Payne et al. 2017). Most of the MTBC organisms have been shown to persist at aggregation points, like wallowing sites and watering holes, for up to ten days (Barasona et al. 2017). Like feral pigs, warthogs are frequently seen scavenging (Nugent et al. 2002, pers. comm. Dr Peter Buss, 2016). Therefore, warthogs could potentially play a similar role in the African ecosystem as their European cousins.

To date, no formal study has investigated the role, epidemiology, and occurrences of *M. bovis*-infection in warthogs. Moreover, there are currently no validated ante-mortem tests available for detection of infection in this species. The present study aimed to develop novel

assays, investigate the occurrence and prevalence in affected populations, and characterize the *M. bovis* strains isolated from infected warthogs.

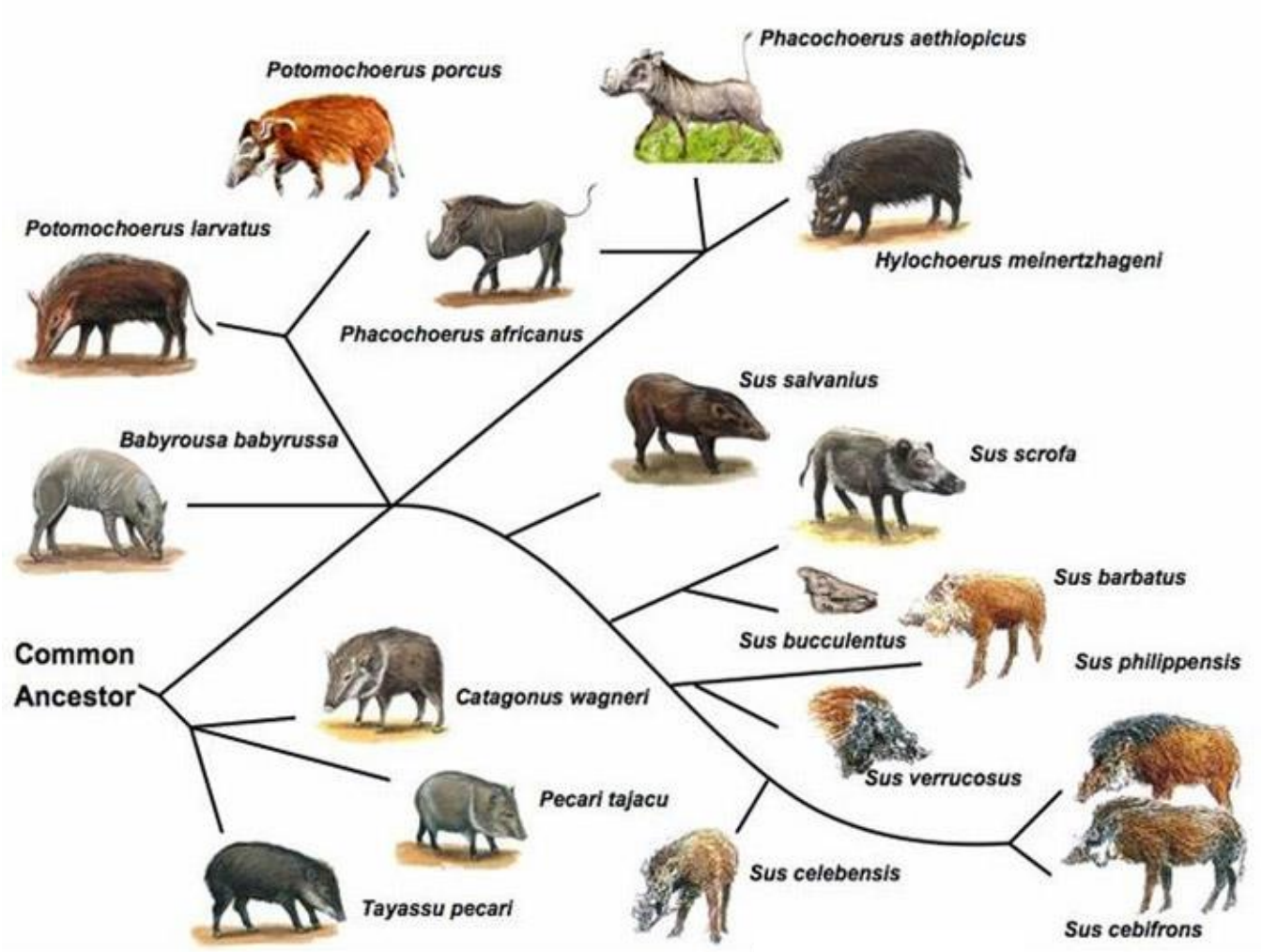


**Figure 1.6** The phylogenetic relationship of the Suiform. Indicating the relatedness of warthogs. Adapted from Chen et al. (2007)

The ante-mortem assays that were evaluated during this study included serological tests, such as the indirect purified protein derivative (PPD) enzyme linked immunosorbent assay (ELISA), TB ELISA-VK® and DPP® VetTB; cell-mediated immunoassays such as cytokine production (i.e., interferon-gamma and IP-10) and cytokine gene expression assays (i.e. *IFNG, TNFA, CXCL9, CXCL10* and *CXCL11*) (Thom et al. 2006; Casal et al. 2014; Goosen

et al. 2014b). Spoligotyping and WGS was used to describe the molecular epidemiology of *M. bovis* isolated from warthogs. These tools will allow further investigation of the role that warthogs play in the epidemiology of bTB in the ecosystem.

## Research question, study aims and objectives

***Research question:***

What is the prevalence and molecular epidemiology of *M. bovis*-infections in warthogs?

***Aim:***

To characterise the prevalence and molecular epidemiology of *M. bovis* infections in warthogs.

***Objectives***:

1. To define reference cohorts of *M. bovis* infected and uninfected warthogs.
2. To develop and evaluate humoral diagnostic assays that can distinguish between *M. bovis*-infected and uninfected warthogs.
3. To develop and evaluate cytokine release assays that can distinguish between *M. bovis*-infected and uninfected warthogs.
4. To develop a cytokine gene expression assay that can distinguish between *M. bovis*-infected and uninfected warthogs.
5. To measure the prevalence of *M. bovis*-infection in selected warthog populations.
6. To genetically characterize *M. bovis* strains isolated from warthogs.
7. To compare genotypes of *M. bovis* strains isolated from warthogs with those isolated from other animal species in South Africa.

## Originality of study

To understand the impact a species has on the epidemiology of a disease, such as bTB, species specific and accurate diagnostic assays are needed. Improved understanding of the disease dynamics would facilitate management of bTB, lead to new insights for conservation efforts and provide necessary tools for routine disease surveillance in complex ecosystems. This is especially true for species, such as warthogs, that could serve as disease sentinels as they are abundant and may have less economic value. However, the lack of appropriate reagents means that diagnostic assays are often unavailable in these species. Furthermore, warthogs are an underappreciated species in the disease ecology of bTB, as they are generally seen as a dead-end host.

This study describes the development and use of immunologic and molecular assays for detection of *M. bovis*-infection in warthogs. This is achieved by adapting and optimising assays that target the host's cell-mediated and humoral immune responses. As mycobacterial infections are not well described in warthogs, this study further identified various species of *Mycobacterium* and strains of *M. bovis* that circulate within various populations. These findings will help elucidate the epidemiological role of warthogs as potential disease reservoirs in their ecosystems and their potential use as a disease sentinels for bTB.

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# Chapter 2

## Test performance of three serological assays for the detection of *Mycobacterium bovis* infection in common warthogs (*Phacochoerus africanus*)

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**My contribution to this research article:**

Planning of project
Blood collection
Running all assays
Post mortem examinations
Tissue sample collection
Mycobacterial culturing
Speciation by PCR
Data interpretation
All statistical analyses
Writing of manuscript

**Abstract**

Sporadic cases of bovine tuberculosis (bTB) have been reported in warthogs in southern Africa and confirmed through mycobacterial culture. However, there are no validated ante-mortem tests currently available for bTB in warthogs. In this study, we evaluated the use of three serological assays for the detection of *Mycobacterium bovis* infection in warthogs; an indirect enzyme-linked immunosorbent assay (ELISA) using bovine purified protein derivative ($PPD_b$) as a capture antigen (indirect PPD ELISA), as well as two commercial assays, the TB ELISA-VK® and DPP® VetTB Assay. Test performance of these assays was compared using sera from 35 warthogs of known *Mycobacterium bovis* infection status. All three assays were able to distinguish *M. bovis*-infected from uninfected individuals with high sensitivity (Se) and specificity (Sp) (indirect PPD ELISA Se: 88%, Sp: 89%; TB ELISA-VK® 88%, 79%; DPP® VetTB Assay 75%, 89%, respectively). The assays performed very similarly, and the ELISA assays showed the greatest agreement ($\kappa = 0.89$). These results indicate that *M. bovis*-infected warthogs develop measurable pathogen-specific humoral responses which can be used to distinguish them from uninfected animals. Therefore, serological assays have value as ante-mortem bTB diagnostic tests in warthogs.

**Highlights:**

- Serology can distinguish between *M. bovis*-infected and uninfected warthogs.
- Three serological assays showed high test agreement.
- High sensitivity and specificity were achieved with the three serological assays.
- Serology has value as a diagnostic tool for detection of *M. bovis* infection in warthogs.

Keywords: Bovine tuberculosis, DPP® VetTB test, *Mycobacterium bovis*, PPD ELISA, TB ELISA-VK®, Warthog

## Introduction

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis*, was introduced to South Africa with the importation of European cattle in the 18th century (Michel et al. 2006). Bovine TB has become endemic in some areas of South Africa and it has been confirmed in 17 animal species, including common warthogs (*Phacochoerus africanus*) (Michel et al. 2006; Hlokwe et al. 2014). Although warthogs have been considered spill-over hosts for bTB, they have the potential to become reservoir hosts under certain circumstances, i.e. increased population densities (de Lisle et al. 2002; De Garine-Wichatitsky et al. 2013). Native Eurasian wild boar (*Sus scrofa*), which occupy a similar ecological niche to warthogs, are known to serve as reservoir hosts of *M. bovis* in wildlife populations in the Iberian Mediterranean ecosystem (Parra et al. 2003; Martín-Hernando et al. 2007; Naranjo et al. 2008). The presence of these reservoir hosts has resulted in interspecies spread (i.e., between wild boar and red deer) of bTB through shared pastures and watering holes (Barasona et al. 2014; Naranjo et al. 2008). Warthogs might play a similar role in South Africa.

Warthogs, as with other wild suids, are difficult to keep within artificial barriers, which may increase the risk of disease spread (Michel et al. 2006; Renwick et al. 2007). Movement of warthogs across fences increases potential wildlife-livestock interactions, especially adjacent to national park and game reserve borders (Musoke et al. 2015). In addition to being a livestock health threat and an economic loss to neighbouring cattle farmers, there is a potential zoonotic health risk facing residents bordering these parks and reserves.

There is currently limited knowledge regarding the *M. bovis* infection status of warthogs in South Africa and the epidemiological consequences for other species. Typically, *M. bovis* is detected in warthogs by post-mortem examination, mycobacterial culture of tissue, and

pathological evaluation of lesions (Michel et al. 2006; Hlokwe et al. 2014). However, these techniques are time consuming and expensive (Cardoso-Toset et al. 2015). Therefore, it is essential to validate ante-mortem tests for the detection of *M. bovis* in warthogs as tools for epidemiological studies, disease management and surveillance.

Serology has been successfully used to detect *M. bovis* infection in multiple species (Maas et al. 2013; Bezos et al. 2014). Serological assays provide rapid results and have been employed for *M. bovis* surveillance in wild boar and free-ranging pigs (Aurtenetxe et al. 2008; Boadella et al. 2011; Cardoso-Toset et al. 2015; Che' Amat et al. 2015). Although no validated tests exist for warthogs, a recent pilot study suggests that the DPP® VetTB assay may be useful for detection of *M. bovis* infection in this species (Miller et al. 2016). Validated serological tests will provide tools for ante-mortem disease testing of individual warthogs in zoos and other captive populations, as well as disease surveillance, translocation, and research (Chambers 2013; Maas et al. 2013; Broughan et al. 2013).

Thus, the aim of this study was to evaluate test performance of an indirect enzyme-linked immunosorbent assay (ELISA) using bovine purified protein derivative ($PPD_b$) as a capture antigen (indirect PPD ELISA) as well as two commercial assays, the TB ELISA-VK® and DPP® VetTB assay for the ante-mortem detection of *M. bovis*-infected warthogs.

## Materials and Methods

### *Animals and sampling*

Between 2013 and 2015, opportunistic samples were obtained from 35 warthogs that were euthanized for management purposes. These animals were captured in four localities in South Africa; uMhkuze Nature Reserve in Kwa-Zulu Natal (n=9), Marloth Park on the southern

border of the Kruger National Park (KNP) in Mpumalanga (n=12), as well as within the KNP in the vicinity of Skukuza (n=6) and Satara (n=8).

Blood was collected into Vacutainer® Serum Tubes (BD Biosciences, Franklin Lakes, NJ, USA), and serum was harvested and stored at -80°C until analysed. Complete post-mortem examinations were performed on all 35 animals. Head, cervical, thoracic, abdominal and peripheral lymph nodes were examined for the presence of gross lesions consistent with bTB. Representative samples from lymph nodes were collected from all warthogs for mycobacterial culture. In cases where no visible lesions were observed, lymph node samples were pooled according to anatomical sample site, i.e. head and neck, thorax, abdomen and the periphery. Separate samples were collected from any tissue with visible TB-like lesions (VL). 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).

*Mycobacterial cultures and speciation*

Lymph node pools and tissues with VL were processed for mycobacterial culture using the BACTEC™ MGIT™ 960 system (BD Biosciences) as previously described (Goosen et al. 2014). 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). Culture results were used to define animals as *M. bovis*-infected or uninfected.

*Indirect PPD ELISA*

The indirect PPD ELISA was based on other published protocols, with the following modifications (Wiker et al. 1998; Lilenbaum et al. 1999; Aurtenetxe et al. 2008). Briefly, test

wells were prepared with 100µl of 10 µg/ml $PPD_b$ (Prionics, Schlieren-Zurich, Switzerland) in carbonate buffer and the control wells contained 100µl of blocking buffer (BB) consisting of 20% fat-free milk (Clover, Roodepoort, South Africa) and 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS) (Lonza, Verviers, Belgium). Plates were incubated at 37°C for 1h then washed. Hereafter, wells were blocked and incubated for 1h at room temperature (RT) and washed. Serum was diluted 1:100 in BB for each animal as done by Lilenbaum et al. (1999), 100µl of the diluted sample was added to test and control wells, in duplicate, and incubated for 1h at RT. After washing, peroxidase-conjugated recombinant protein A/G (Thermo Scientific, Hudson, NJ, USA) diluted 1:20000 in PBS was added (100 ul/well) and then incubated for 1h at RT. Hereafter, 100µl of TMB reagent (BD Biosciences, Woodmead, South Africa) was added to washed wells and incubated for 15-20min before adding 50µl of 2M $H_2SO_4$. Absorbance was read at 450nm and 630nm (450nm - 630nm = optical density (OD) result). For each animal, test results were calculated by subtracting the control well OD from the test well OD.

*TB ELISA-VK®*

Serum samples were tested using the commercial TB ELISA-VK® kit (Vacunek, Bizkaia, Spain) according to the manufacturer's recommendations. Optical density values were recorded as well as test results, calculated as the ELISA-Index (EI). The EI was calculated using the kits' prescribed formula and positive control (PC) provided in the kit (Avg. $OD_{405-450}$ Sample / Avg. $OD_{405-450}$ PC).

*DPP® VetTB Assay for Cervids*

Sera were tested using the DPP® VetTB Assay for Cervids (Chembio Diagnostic Systems, Inc., Medford, NY, USA) (DPP® VetTB), according to the manufacturer's instructions. To

quantitate test results as relative light units (RLU), an optical reader (Chembio) was used to measure the reflectance of a control line and two test lines consisting of the antigens MPB83 (line 1) and ESAT-6/CFP-10 recombinant protein (line 2). A diagnostic cut-off value of 5.0 RLU was used. Reactivity to either test line in the DPP® VetTB was considered as a positive result.

*Statistical analysis*

Manta PC Software (Dazdaq Ltd, Brighton, England) was used to gather the optical density data, which was exported into a Microsoft Excel (2013) worksheet. Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, March 2007) and MedCalc version 16 (MedCalc Software, Ostend, Belgium). Assay results of *M. bovis*-infected and uninfected warthogs were compared using the unpaired Student's *t*-test. Receiver operator characteristic (ROC) curve analysis was used to determine a cut-off value for the indirect PPD ELISA, using the Youden's index. ROC was furthermore used to compare the test performance of all three assays as well as calculating warthog-specific cut-offs. Test agreement between assays was calculated as the Cohen's kappa coefficient using GraphPad Software (http://graphpad.com/quickcalcs/kappa1/). Correlations between assays were calculated using the Spearman rank test. For this analysis, OD values were used for the ELISA based assays, and the RLU values of the DPP® VetTB were log transformed. Statistical significance was set at p-value $< 0.05$.

**Results**

The diagnostic test and mycobacterial culture results for individual warthogs are summarized in Table 2.1. Of 35 warthogs tested, 16 (46%) were confirmed to be infected with *M. bovis* while non-tuberculous mycobacteria (NTM) were isolated from lymph nodes of 9/19 (47%)

*M. bovis*-uninfected animals. The majority of *M. bovis*-infected warthogs, i.e. 15/16 (94%), had VL, compared to 4/19 (21%) uninfected individuals.

For the indirect PPD ELISA, test values for *M. bovis*-infected warthogs (mean OD 1.76 ± SD 0.59) were significantly greater than those for uninfected animals (mean OD 0.78 ± SD 0.54) (Fig. 2.1A). With an optimal cut-off value of 1.06, this assay correctly categorized 14/16 infected warthogs with a sensitivity (Se) of 88% (95% CI: 62-98%) and 17/19 uninfected animals with a specificity (Sp) of 89% (95% CI: 67-99%) (Fig. 2.2).

In the case of the TB ELISA-VK® kit, test values for *M. bovis*-infected warthogs (mean OD 0.68 ± SD 0.40) were also significantly greater than those of the uninfected animals (mean OD 0.19 ± SD 0.21) (Fig. 2.1B). The cut-off set by the manufacturer for the TB ELISA-VK® kit assay (EI ≥ 0.200) correctly categorized 14/16 infected animals (Se: 88%; 95% CI: 62-98%) and 15/19 uninfected animals (Sp: 79%; 95% CI: 54-94%). Using ROC curve analysis, a warthog-specific cut-off was calculated as OD = 0.23 (Fig. 2.2, Table 2.2).

The DPP® VetTB assay values for *M. bovis*-infected animals (mean RLU 46.34 ± SD 49.31) were once again significantly greater than those of the uninfected warthogs (mean RLU 5.59 ± SD 16.31) (Fig. 2.1C). The reader cut-off set by the manufacturer (RLU = 5.0) correctly categorized 12/16 infected animals (Se: 75%; 95% CI: 48-93%) and 17/19 uninfected warthogs (Sp: 89%; 95% CI: 67-99%). Using ROC curve analysis, a warthog-specific cut-off

**Table 2.1** Summary of results for three serological assays and mycobacterial cultures from 35 opportunistically sampled warthogs.

| Animal ID | Indirect PPD ELISA | | TB ELISA-VK® | | DPP® VetTB Assay | | Bacterial Culture | Pathology |
|---|---|---|---|---|---|---|---|---|
| | Result[a] | ΔOD (450nm) | Result | EI[b] | Result | RLU[c] | | |
| **13/535** | P | 2.2 | P | 1.46 | P | 55.7 | *M. bovis* | VL[e] |
| **14/001** | P | 2.1 | P | 1.48 | P | 131.2 | *M. bovis* | ND[f] |
| **14/227** | P | 2.1 | P | 1.06 | P | 72.6 | *M. intracellulare, M. simiae* | ND |
| **14/335** | N | 1.0 | P | 0.21 | N | 3.0 | *M. intracellulare, M. simiae* | NVL[g] |
| **15/137** | N | 0.3 | N | 0.15 | N | 0.0 | NMC[d] | NVL |
| **15/138** | N | 0.6 | N | 0.15 | N | 3.2 | NMC | NVL |
| **15/249** | P | 1.8 | P | 1.16 | P | 21.4 | *M. bovis* | VL |
| **15/250** | P | 2.0 | P | 0.58 | P | 54.3 | *M. bovis* | VL |
| **15/251** | P | 1.1 | N | 0.16 | N | 4.8 | *M. bovis* | VL |
| **15/262** | N | 0.9 | P | 0.28 | N | 3.0 | NMC | VL |
| **15/264** | P | 1.7 | P | 0.68 | P | 12.0 | *M. bovis* | VL |
| **15/265** | P | 1.9 | P | 0.76 | P | 37.3 | *M. bovis* | VL |
| **15/266** | P | 2.2 | P | 1.30 | P | 88.5 | *M. bovis* | VL |
| **15/267** | P | 2.2 | P | 1.45 | P | 58.3 | *M. bovis* | VL |
| **15/268** | P | 1.5 | P | 0.32 | N | 0.0 | *M. bovis* | VL |
| **15/269** | P | 1.9 | P | 0.62 | P | 15.6 | *M. bovis* | VL |
| **15/270** | P | 2.2 | P | 1.61 | P | 155.5 | *M. bovis* | VL |
| **15/271** | N | 0.5 | N | 0.09 | N | 3.8 | NMC | VL |
| **15/513** | P | 2.4 | P | 1.00 | P | 7.6 | *M. bovis* | VL |
| **15/514** | N | 0.6 | N | 0.20 | N | 0.0 | *M. simiae* | NVL |
| **15/515** | N | 0.6 | N | 0.18 | N | 0.0 | *M. intracellulare* | NVL |
| **15/516** | N | 0.3 | N | 0.13 | N | 2.8 | *M. asiaticum* | NVL |
| **15/517** | N | 0.5 | P | 0.21 | N | 0.0 | *M. bovis* | NVL |
| **15/534** | N | 0.7 | N | 0.16 | N | 3.1 | *M. simiae* | NVL |
| **15/535** | N | 0.9 | N | 0.16 | N | 3.2 | *M. paraffinicum, scrofulaceam* | NVL |
| **15/536** | N | 0.5 | N | 0.16 | P | 5.7 | *M. simiae* | NVL |
| **15/W/002** | N | 0.7 | N | 0.14 | N | 0.0 | NMC | VL |
| **15/W/004** | N | 0.5 | N | 0.13 | N | 0.0 | NMC | NVL |
| **15/W/007** | N | 0.3 | N | 0.12 | N | 0.0 | *M. intracellulare* | VL |
| **15/W/009** | P | 2.3 | P | 0.83 | N | 0.0 | NMC | NVL |
| **15/W/010** | N | 0.6 | N | 0.15 | N | 0.0 | NMC | NVL |
| **15/W/012** | N | 0.7 | N | 0.14 | N | 3.0 | NMC | NVL |
| **15/W/013** | N | 0.6 | N | 0.15 | N | 2.8 | NMC | VL |
| **15/W/015** | N | 0.4 | N | 0.12 | N | 0.0 | *M. bovis* | VL |
| **15/W/018** | P | 2.0 | P | 0.72 | P | 99.2 | *M. bovis* | VL |
| **Total Positive** | 16 | | 18 | | 14 | | 16 | 19 VL |

[a] P = Positive, N = Negative
[b] ELISA Index value
[c] Relative light units
[d] Negative mycobacterial culture
[e] Visible bTB-like lesion
[f] Not done
[g] No visible bTB-like lesion

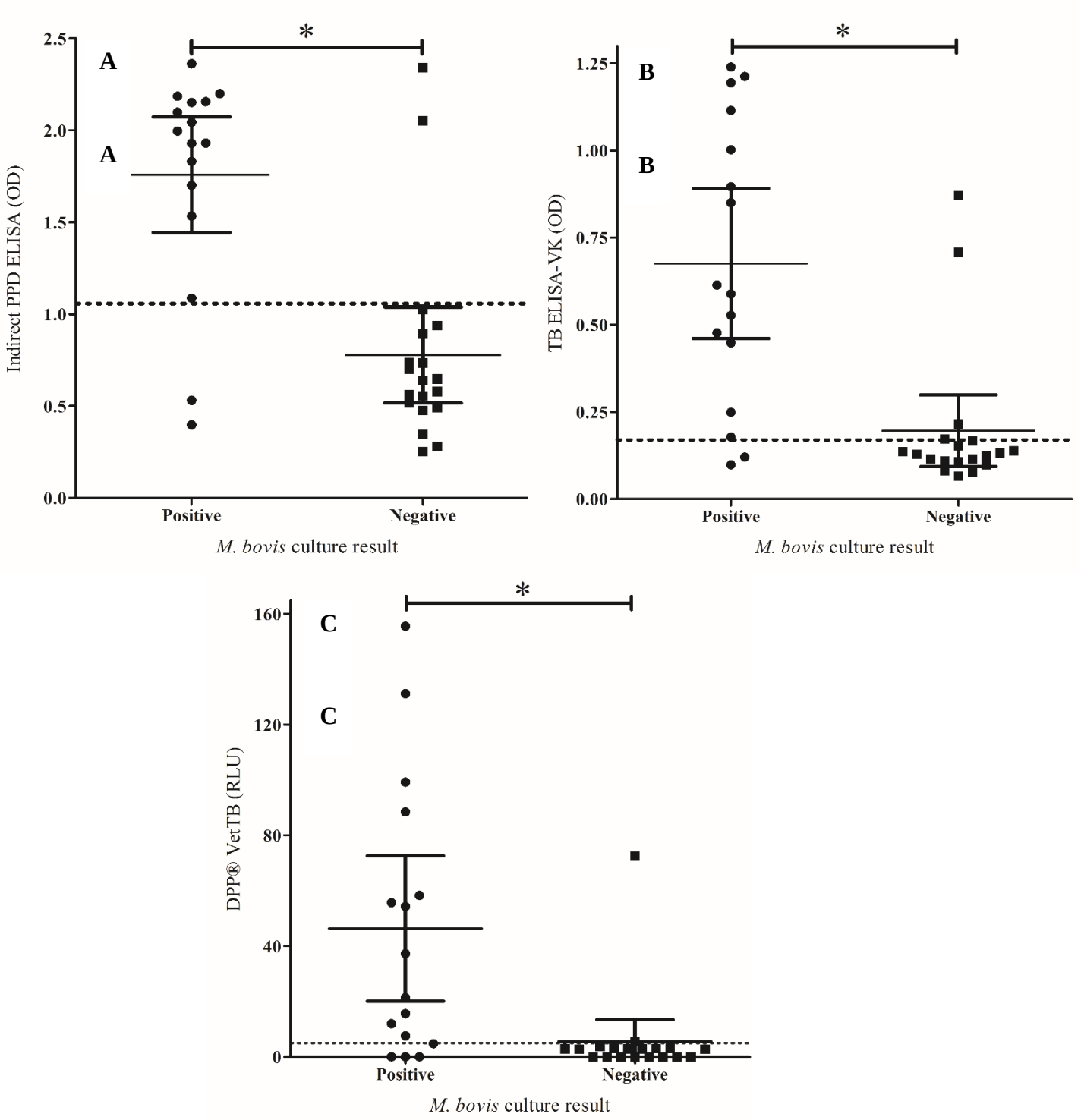


**Figure 2.1** Test values for the indirect PPD ELISA (A), TB ELISA-VK® (B) and DPP® VetTB (C) tests for M. bovis-infected and uninfected warthogs. Mean and 95% confidence interval bars are shown. Dotted lines represent the calculated warthog-specific cut-off from the ROC curve analysis. OD – optical density; RLU – relative light units. *p<0.05 (Unpaired Student t-test)

was calculated as RLU = 4.3 (Fig. 2.2, Table 2.2). Among the *M. bovis*-infected warthogs, there was a higher rate of seroreactivity to MPB83 with 12/16 positive samples (75%), compared to ESAT-6/CFP10 recombinant protein with 7/16 positive samples (44%). Of these, 7/16 (44%) samples were seroreactive at both test lines.

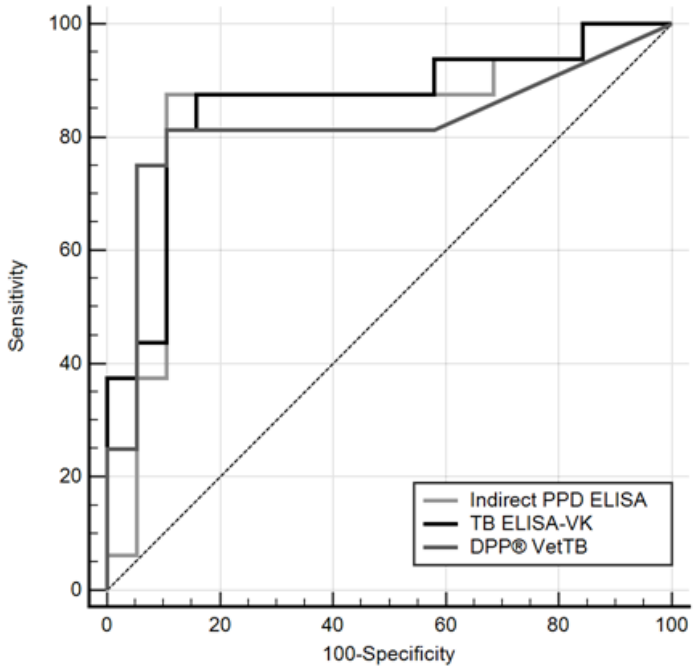


**Figure 2.2** ROC curve analysis of the indirect PPD ELISA, TB ELISA-VK® and DPP® VetTB test results based on M. bovis culture results.

**Table 2.2** Sensitivity and specificity (95% confidence interval) of the TB ELISA-VK® and DPP® VetTB assays in warthogs using the recommended manufacturer cut-off value and a warthog specific cut-off value.

| | **Recommended manufacturer assay** | | | **Warthog specific assay** | | |
|---|---|---|---|---|---|---|
| | **Cut-off** | **Sensitivity** | **Specificity** | **Cut-off** | **Sensitivity** | **Specificity** |
| **TB ELISA-VK®** | 0,200[a] | 88% (62-98%) | 79% (54-94%) | 0,175[a] | 88% (62-98%) | 84% (60-97%) |
| **DPP® VetTB** | 5,0[b] | 75% (48-93%) | 89% (67-99%) | 4,3[b] | 81% (54-96%) | 89% (67-99%) |

[a] Optical density
[b] Relative light units

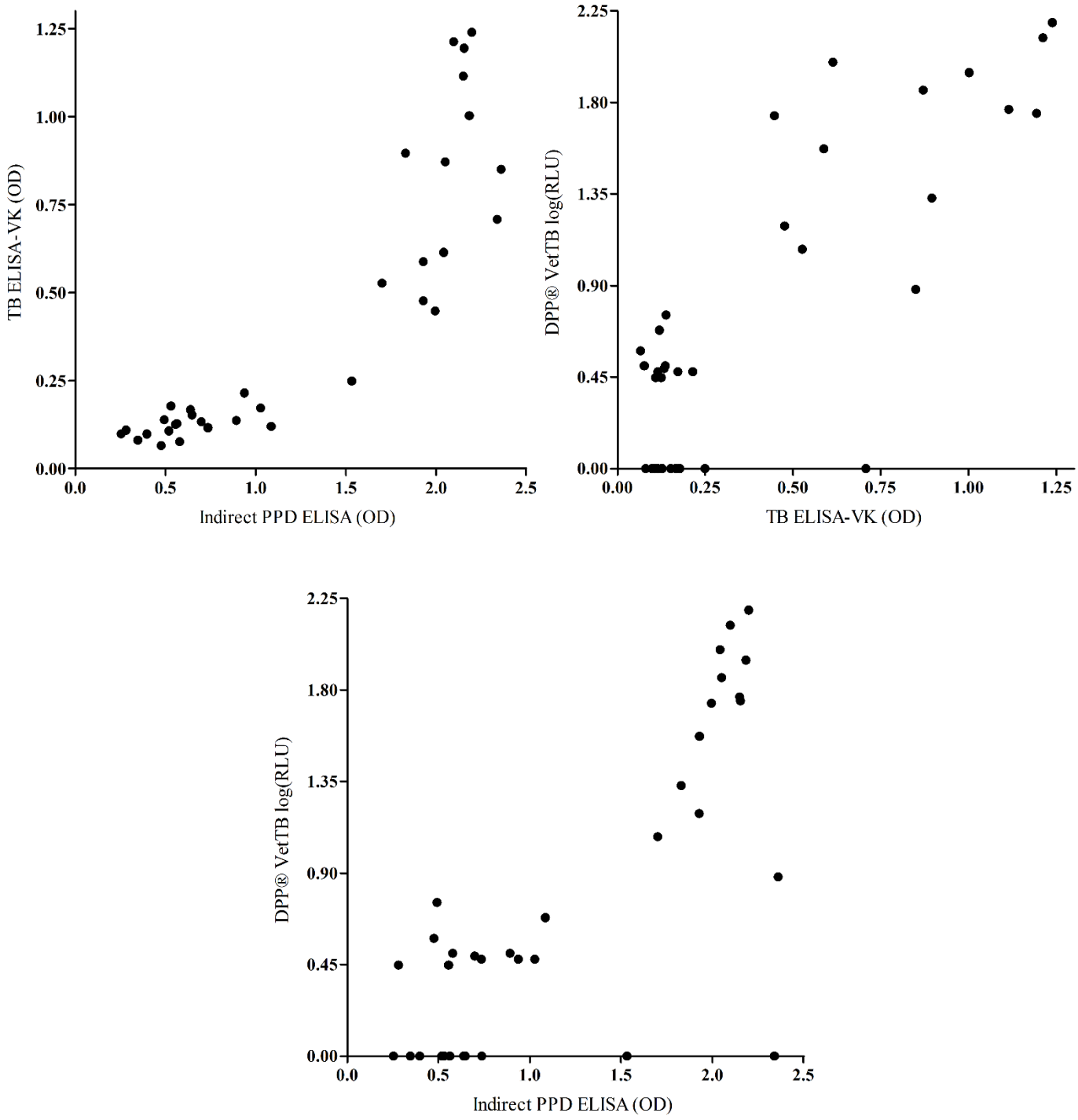


**Figure 2.3** Correlation between the indirect PPD ELISA, TB ELISA-VK® and DPP® VetTB test values. *p<0.05 (Spearman ranked test)

Test agreement and correlation of assay values were assessed (Fig. 2.3, Table 2.3). Test values for the indirect PPD ELISA and TB ELISA-VK® showed a very good correlation ($r = 0.89$; $p < 0.001$) (Fig. 2.3A). There was good correlation between values for the DPP® VetTB assay and both the indirect PPD ELISA and TB ELISA-VK® ($r = 0.69$ and 0.68, respectively; $p < 0.001$) (Fig. 2.3B, 2.3C). The indirect PPD ELISA and TB ELISA-VK® showed greatest test agreement ($\kappa = 0.89$), with lesser agreement between the DPP® VetTB assay and either ELISA ($\kappa = 0.66$). No significant differences were observed when comparing ROC curves for each assay (Z-score < 1.96) (Fig. 2.2).

Of the *M. bovis*-uninfected warthogs, five animals tested positive with one or more serological assays. Of these, NTMs were isolated from the lymph nodes of three animals (Table 2.1).

**Table 2.3** Test agreement between serological assay results from 35 warthogs using the Cohen's Kappa coefficient (κ) (95% confidence interval).

| | **Indirect PPD ELISA** | **TB ELISA-VK®** | **DPP® VetTB** |
|---|---|---|---|
| **Indirect PPD ELISA** | 1 | 0.89 (0.73-1.00) | 0.66 (0.42-0.90) |
| **TB ELISA-VK®** | 0.89 (0.73-1.00) | 1 | 0.66 (0.42-0.90) |
| **DPP® VetTB** | 0.66 (0.42-0.90) | 0.66 (0.42-0.90) | 1 |

## Discussion

Our results demonstrate that warthogs develop a humoral response to *M. bovis* infection that is detectable using the indirect PPD ELISA, the TB-ELISA VK® assay and the DPP® VetTB assay. These assays could discriminate between *M. bovis*-infected and -uninfected animals with high Se and Sp. Additionally, they showed good agreement, supporting the use of serological assays for the detection of bTB in warthogs.

Both ELISA-based serological assays used in this study had similar test performance. A small variation in Se and Sp was observed between the two assays, although this difference was insignificant. Slight changes in test performance could be due to different sources, variable composition or concentration of $PPD_b$ used (Schiller et al. 2010). Another difference could be detection methods. The TB ELISA-VK® used protein G only, compared to the indirect PPD ELISA that used a recombinant protein A/G. Even though protein G has a higher affinity for antibodies in most species (Harboe et al. 1990), the addition of protein A might decrease the detection threshold. However, the accuracy of the indirect PPD ELISA and the TB-ELISA VK® assay appeared to be essentially the same in this study.

In the current study, the DPP® VetTB had slightly lower Se and higher Sp than the ELISAs, although these differences were not statistically significant, for either the manufacturer or species-specific cut-off. Mycobacterial antigens used to detect antibodies differ between these assays, which could contribute to the differences observed. The ELISA assays incorporate a broad range of mycobacterial antigens (PPD) whereas the DPP® VetTB utilizes limited specific antigens (MPB83 and ESAT-6/CFP10). Including more antigens with shared epitopes would explain the slightly higher Se and lower Sp in the ELISA assays compared to the DPP® VetTB assay.

Identifying immunodominant antigens in a species is an important consideration for bTB diagnostic test development and application. The selection of antigens and species-specific responses will impact assay performance. Indirect PPD ELISAs have reported low Se or Sp in badgers, cattle and reindeer (Maas et al. 2013). In contrast, other species such as elephants, deer, llamas, and wild boar develop significant antibody titers during mycobacterial infection, resulting in high Se and Sp in PPD ELISAs and the DPP® VetTB assay (Boadella et al. 2011;

Chambers 2013; Lyashchenko et al. 2008; Maas et al. 2013). This has led to the development of a commercial test kit, the TB ELISA-VK®, that is used to screen wild boar populations and monitor disease spread in the Mediterranean region (Boadella et al. 2011; Richomme et al. 2013). In this study, more warthogs had antibodies to MPB83 compared to ESAT6/CFP10. This outcome has been reported previously in *M. bovis*-infected badgers, deer, possums and wild boar (Lyashchenko et al. 2008; Boadella et al. 2011). Therefore, *M. bovis*-infected warthogs appear to recognize similar immunodominant antigens to wild boar, which supports our hypothesis that serological assays developed for wild boar (Aurtenetxe et al. 2008; Boadella et al. 2011) would have similar test performance in warthogs.

Using the manufacturer's cut-off value, the Se of the TB ELISA-VK® (Se: 88%; 95% CI: 62-98%) and the Sp (Sp: 79%; 95% CI: 54-94%) of the assay for warthogs were similar to those reported in wild boar (Se 73-79%, Sp 84-100% for wild boar) (Aurtenetxe et al. 2008; Boadella et al. 2011). Similarly, the Se and Sp of the DPP® VetTB (Se: 75%; 95% CI: 48-93%; Sp: 89%; 95% CI: 67-99%) were comparable to those for wild boar (Se 88% and Sp 90%) (Boadella et al. 2011).

Tests for bTB should use diagnostic cut-off values that maximize detection of infected animals, while minimizing false positive and negative results. ROC curve analysis generated warthog specific cut-off values for the TB ELISA-VK® and DPP® VetTB which were similar to cut-off values recommended by the assay manufacturers (Table 2.2). However, application of the warthog specific cut-off values did not significantly change the sensitivity and specificity of the DPP® VetTB and TB ELISA-VK® assays.

Advantages of serological assays are that they are relatively inexpensive, they can be used to screen large numbers of samples, and they can detect antibodies to a broad range of mycobacterial antigens (Chambers 2009, 2013). Serological assays are also ideal tools for retrospective detection of bTB in different species and geographical ranges (Chambers 2009; Richomme et al. 2013). ELISA based tests are ideal disease surveillance tools due to the high through-put (Boadella et al. 2011; Maas et al. 2013). The advantage of using an in-house PPD ELISA is the similar Se and Sp to the commercial kits and reagents are available through local suppliers. In contrast, the commercial test kits are not readily available in South Africa and require specific importation. Therefore, the indirect PPD ELISA is a suitable alternative for bTB testing of warthogs, eliminating some of the costs and logistical challenges associated with imported kits. A specific advantage of the rapid DPP® VetTB assay is that it is well-suited for field testing animals prior to translocation (Boadella et al. 2011; Chambers 2013; Maas et al. 2013). However, none of the tests used in this study are currently validated for warthogs.

Five seroreactive warthogs were not confirmed to be *M. bovis* infected by culture. One potential cause for this finding could be limitations in sensitivity of mycobacterial cultures, leading to false negative culture results (de la Rua-Domenech 2006; Müller et al. 2011). Alternatively, infection with non-tuberculous mycobacteria and the immunological cross-reactivity to *M. bovis* antigens could result in decreased specificity (Schiller et al. 2010; Maas et al. 2013). Since the study animals originated from opportunistic samples from known *M. bovis*-infected populations, further studies are needed using warthogs from known bTB-free populations to determine true specificity of these serological assays.

In conclusion, results from this study demonstrate that warthogs, similar to other wild suids, develop strong humoral responses during infection with *M. bovis*. This observation supports the application of serological tools for diagnosis, disease surveillance, epidemiological studies, and investigations of the role of bTB in warthogs. However, further studies are needed to validate these diagnostic tools under field conditions.

## Conflict of interest

There is no conflict of interest to be declared by the authors regarding this publication.

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# Chapter 3

## Seroprevalence of *Mycobacterium bovis* infection in warthogs (*Phacochoerus africanus*) in bovine tuberculosis-endemic regions of South Africa

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**My contribution to this research article:**

Planning of project
Blood collection
Running all assays
Post mortem examinations
Tissue sample collection
Mycobacterial culturing
Speciation by PCR
Data interpretation
All statistical analyses
Writing of manuscript

**Abstract**

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis (M. bovis),* has been reported in many species including suids. Wild boar are important maintenance hosts of the infection with other suids, i.e. domestic and feral pigs, being important spill-over hosts in the Eurasian ecosystem. In South Africa, warthogs (*Phacochoerus africanus*) may play a similar role in *M. bovis*-endemic areas. However, novel diagnostic tests for warthogs are required to investigate the epidemiology of bTB in this species. Recent studies have demonstrated that serological assays are capable of discriminating between *M. bovis*-infected and uninfected warthogs (Roos et al. 2016). In the present study, an indirect ELISA utilizing *M. bovis* purified protein derivative (PPD) as a test antigen was used to measure the prevalence and investigate risk factors associated with infection in warthogs from uMhkuze Nature Reserve and the southern region of the Greater Kruger National Park (GKNP). There was a high overall seroprevalence of 38%, with adult warthogs having a higher risk of infection (46%). Seroprevalence also varied by geographic location with warthogs from Marloth Park in the GKNP having the greatest percentage of positive animals (63%). This study indicates that warthogs in *M. bovis*-endemic areas are at high risk of becoming infected with mycobacteria. Warthogs might present an under-recognized disease threat in multi-species systems. They might also serve as convenient sentinels for *M. bovis* in endemic areas. These findings highlight the importance of epidemiological studies in wildlife to understand the role each species plays in disease ecology.

**Highlights:**

- There is a high overall seroprevalence in warthogs from bTB endemic regions.
- Warthogs from Marloth Park had the highest seroprevalence of 63%.

- Both the indirect PPD ELISA and TB ELISA-VK® can be used for surveillance of bTB in warthogs.

Keywords: bTB prevalence, *Mycobacterium bovis*, PPD ELISA, serological assay, warthog

**Introduction**

Bovine tuberculosis (bTB) is a disease caused by infection with *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex (O'Reilly and Daborn 1995). It has become endemic in many parts of the world, including game parks in South Africa, with confirmed cases in more than 17 domestic and wildlife species (Hlokwe et al. 2014; Musoke et al. 2015). Although the bTB status at some locations is known, many parks and private game reserves have no information on whether or not bTB is endemic on their properties (Hlokwe et al. 2014). Many also keep animals with high economic value and endangered species which could be threatened by the presence of disease.

The lack of available diagnostic tests for most wildlife species contributes to the difficulty in determining the bTB status of different populations. Serological assays for bTB reportedly lack the necessary sensitivity of a diagnostic assay (de Lisle et al. 2002). However, Harboe and colleagues (1990) developed a Protein G-based enzyme-linked immunosorbent assay (ELISA) to detect *M. bovis*-specific antibodies in cattle using antigen MPB70. Since then, numerous studies have evaluated ELISA-based assays for bTB detection in animals, but with significant variation in sensitivity and specificity (Maas et al. 2013). For example, serological assays used for badgers (*Meles meles*), possums (*Trichosurus vulpecula*), lions (*Panthera leo*) and buffalo (*Syncerus caffer*) appear to have insufficient sensitivity (23-52%) as diagnostic tools (Maas et al. 2013). However, acceptable sensitivity and specificity of

serological assays have been achieved for multiple cervid species, elephants (*Loxodonta africana* and *Elephas maximus*) and wild boar (*Sus scrofa*) (sensitivity: 72-100%; specificity: 80-100%) (Maas et al. 2013; Broughan et al. 2013). Furthermore, serological tests in other African wildlife species, such as warthogs (*Phacochoerus africanus*), have shown promise as potential diagnostic tools (Miller et al. 2015, 2016).

Recently published research using these tools has demonstrated that humoral immune responses can be used to differentiate between *M. bovis*-infected and uninfected warthogs with high confidence (Roos et al. 2016). The serological assays had high sensitivities (75-88%) and high proportions of test negative animals (79-89%), suggesting that these tests could be used to accurately measure the prevalence of *M. bovis* infection in warthogs (Roos et al. 2016). Serological assays have further potential value in field surveillance since both retrospective and prospective samples can be used (Boadella et al. 2011; Chambers 2013; Maas et al. 2013). Determining prevalence and risk factors will contribute to our understanding of the role that warthogs play in the ecosystem with regards to bTB (Boadella et al. 2011; Roos et al. 2016). Identification of potential maintenance hosts is crucial to inform management and control strategies. In addition, species that may serve as disease sentinels are important in surveillance plans. However, to date no information is available on the prevalence of bTB in warthogs in South Africa.

It is important to determine the prevalence of bTB in warthogs as they are a free-living species in bTB-endemic areas of South Africa, with regular movement between farms and reserves, potentially increasing the intra-population spread of bTB when warthogs cross these fenced barriers (Vicente et al. 2013). Warthogs are not actively managed by hunting compared to many other species sharing the habitat on game farms or reserves. Warthog

reproduction may also be potentially increased when supplemental feeding is provided in these intensively managed reserves. The increasing population numbers may lead to increased risk of intra-population transmission of bTB, since warthogs are communal burrowers, which may also potentially increase the risk of inter-species transmission of (Naranjo et al. 2008; Vicente et al. 2013). Therefore, a need exists to understand the bTB dynamics in warthogs, as it will inform management and disease strategies. The aims of this study were to confirm test performance of serological assays for detection of bTB using different warthog populations, and to use these tools to estimate prevalence and identify risk factors for *M. bovis* infection in this species.

## Materials and methods

### *Animals and sampling*

Samples were collected opportunistically from warthogs that were immobilized or euthanized for management purposes between 2013 and 2015 (n = 170). Warthogs were randomly chosen for sampling, as any animals that were located were sampled. These populations are not actively managed, and sampling is only done as part of other routine procedures (i.e. disease surveillance) (SANParks 2006). There was no selection process for age and sex and all animals appeared to be healthy based on physical examination. Free-ranging warthogs were sampled from four populations: uMhkuze Nature Reserve (MK) in Kwa-Zulu Natal, Marloth Park (MP) on the southern border of Kruger National Park (KNP) in Mpumalanga, and near Skukuza (SK) as well as Satara (SA) within KNP. All parks are situated within South Africa's savanna biome (Mucina and Rutherford 2006). The sex and estimated age were recorded for the majority of warthogs. Age classes were categorized as juveniles being less than 1 year of age, sub-adults 1-2 years of age, and adults being older than 2 years. This was based on body size and dentition (Bradley 1972; Spinage and Jolly 1974; Mason 1984).

Blood was collected into vacutainers and serum harvested as previously described (Roos et al. 2016). Post-mortem examinations were performed on a subset of the warthogs from each population; MK (n = 21), MP (n = 24), SK (n = 5) and SA (n = 19). Lymph node tissues from 67 animals were processed for mycobacterial culture as described below. Tissue samples from two animals were contaminated and excluded from the culture results. Ethical approval for this study was received from Stellenbosch University Animal Care and Use Committee (SU-ACUD15-00029).

*Mycobacterial culture and speciation*

Mycobacterial culture from lymph nodes and any organ with lesions was done as per Goosen et al. (2014) and isolates further speciated as previously described (Roos et al. 2016). Any tissue (including individual lymph nodes) with lesions consistent with bTB and each lymph node pool (i.e., head/cervical, thoracic, peripheral, and abdominal) were cultured separately. For this study, infected animals were defined as *M. bovis* culture-positive; those with negative cultures were considered uninfected, but potentially exposed. Furthermore, non-tuberculous mycobacteria (NTM) were speciated by using 16S rDNA PCR amplification and sequencing (Leclerc et al. 2000). These sequences were then screened using the National Center for Biotechnology Information (NCBI) BLAST alignment tool (http://blast.ncbi.nlm.nih.gov) as previously described (Katale et al. 2014).

*Serological assays*

The indirect PPD ELISA and TB ELISA-VK® were used to test warthog serum samples as previously described (Roos et al. 2016). All warthogs were tested using the indirect PPD ELISA, with a subset of 100 animals tested using the TB ELISA-VK®. Briefly, for the

indirect PPD ELISA, wells were coated with 100µl of 10 µg/ml $PPD_b$ (Prionics, Schlieren-Zurich, Switzerland) or blocking buffer (BB) consisting of 20% fat-free milk (Clover, Roodepoort, South Africa) and 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS) (Lonza, Verviers, Belgium) then incubated for 1 hr at 37°C. After washing, blocking buffer was added to the plate and incubated for 1 hr at room temperature (RT). Plates were washed and then a 1:100 dilution of serum in BB was added to test and control wells, in duplicate, and incubated for 1 hr at RT. After washing, peroxidase-conjugated recombinant protein A/G (Thermo Scientific, Hudson, NJ, USA) diluted 1:20000 in PBS was added (100 ul/well) for 1 hr at RT, then washed again. TMB reagent, 100 ul/well (BD Biosciences, Woodmead, South Africa) was added to the plate and incubated for 15-20 min before stopping the reaction with 50µl of 2M $H_2SO_4$. The commercial TB ELISA-VK® kit (Vacunek, Bizkaia, Spain) was performed according to the manufacturer's recommendations using a 1:200 dilution of warthog serum.

Optical density (OD) measurements were taken at different wave-lengths from the two assays as they used different substrates during the detection step. Measurements were performed at 450nm and 630nm for the indirect PPD ELISA assay with the test result being the differential (450nm-630nm), and at 405nm and 450nm for the TB ELISA-VK® (OD result = 405nm-450nm). Cut-off values for the assays were set according to Roos et al. (2016) for the indirect PPD-ELISA (OD $\geq$ 1.04) and to the manufacturer's cut-off for the commercial TB ELISA-VK® (ELISA-Index $\geq$ 0.2).

*Statistical analysis*

Manta PC Software (Dazdaq Ltd, Brighton, England) was used to gather the optical density data from the ELISA plate reader, which were exported into a Microsoft Excel (2013)

worksheet. Statistical analyses for the descriptive statistics were performed using GraphPad Prism version 5 (GraphPad Software, March 2007) and Microsoft Excel (2013). Assay results of *M. bovis*-infected and uninfected warthogs were compared using the unpaired Student's *t*-test. A Kruskal-Wallis with Dunn's Multiple Comparison Test was used to compare inter- and intragroup variation of infection status based on location and age. Test agreement between assays was calculated as the Cohen's Kappa coefficient using GraphPad Software (http://graphpad.com/quickcalcs/kappa1/).

The seroprevalence of different groups (location, age and sex) was described as a percentage of *M. bovis*–infected animals, including 95% confidence interval (CI). These were compared by screening for univariable discrepancies, with standard 2x2 or 2x3 contingency tables. *M. bovis* infection risk was then compared between groups using the Pearson chi-squared test or Fisher's exact test. To account (adjust) for the combined effect of *M. bovis* infection risk, a multivariable logistic regression model was used to compare location, age and sex. The multivariable logistic regression models were performed using Stata 13.1 (StataCorp, College Station, Texas, USA). Statistical significance was set at p-value $< 0.05$.

**Results**

Mycobacterial culture was performed on tissues from a subset of sampled warthogs to determine estimated *M. bovis* infection prevalence and assess serological test performance. Of the 67 warthogs that had lymph nodes and tissues submitted for culture, 25 (37%) warthogs were confirmed as *M. bovis*-infected. Based on these results, assay sensitivity was calculated for the indirect PPD ELISA as 92% (95% CI 75-98%) and TB ELISA-VK® as 86% (95% CI 65-95%). Both assays performed similarly with good test agreement between the indirect PPD ELISA and the TB ELISA-VK® ($\kappa = 0.69$; 95% CI 0.55-0.84).

Using these assays, *M. bovis* seroprevalence was determined using a larger cohort of warthog samples from four populations in South Africa. Overall, 64/170 (38%; 95% CI 31-45%) warthogs were indirect PPD ELISA-positive and 44/100 (44%; 95% CI 35-54%) were TB ELISA-VK®-positive. The seroprevalences using the two tests were not significantly different ($z = -1.029$, $p > 0.05$). When both assays were used to determine the seroprevalence, 49/100 (49%; 95% CI 39-59%) warthogs tested positive in at least one of the two assays.

To determine whether there was any impact of NTM infections on the serological results, OD values from *M. bovis* culture-positive, *M. bovis*-negative/NTM-positive, and uninfected warthogs were compared to one another. NTM species isolated from warthogs are shown in Table 3.1. The median indirect PPD ELISA OD value from *M. bovis* culture-positive animals (*Mdn* = 1.97) was significantly greater than *M. bovis*-negative/NTM-positive (*Mdn* = 0.67) or uninfected (*Mdn* = 0.52) warthogs ($p < 0.001$), whereas no significant difference was observed between the latter two groups (Fig. 3.1). One warthog was co-infected with *M. bovis* and *M. simiae* and thus considered as *M. bovis*-infected, with an indirect PPD ELISA OD value of 2.16. None of the NTM infected warthogs were co-infected with other NTMs.

**Table 3.1** List of non-tuberculous mycobacteria (NTM) isolated from *M. bovis* culture-negative warthogs.

| **Mycobacteria isolated** | **N**† |
|---|---|
| *M. asiaticum* | 2 |
| *M. intracellulare* | 2 |
| *M. parascrofulaceam* | 2 |
| *M. scrofulaceum* | 2 |
| *M. simiae* | 8 |
| *M. avium* | 6 |

† Number of warthogs with specific NTM isolated

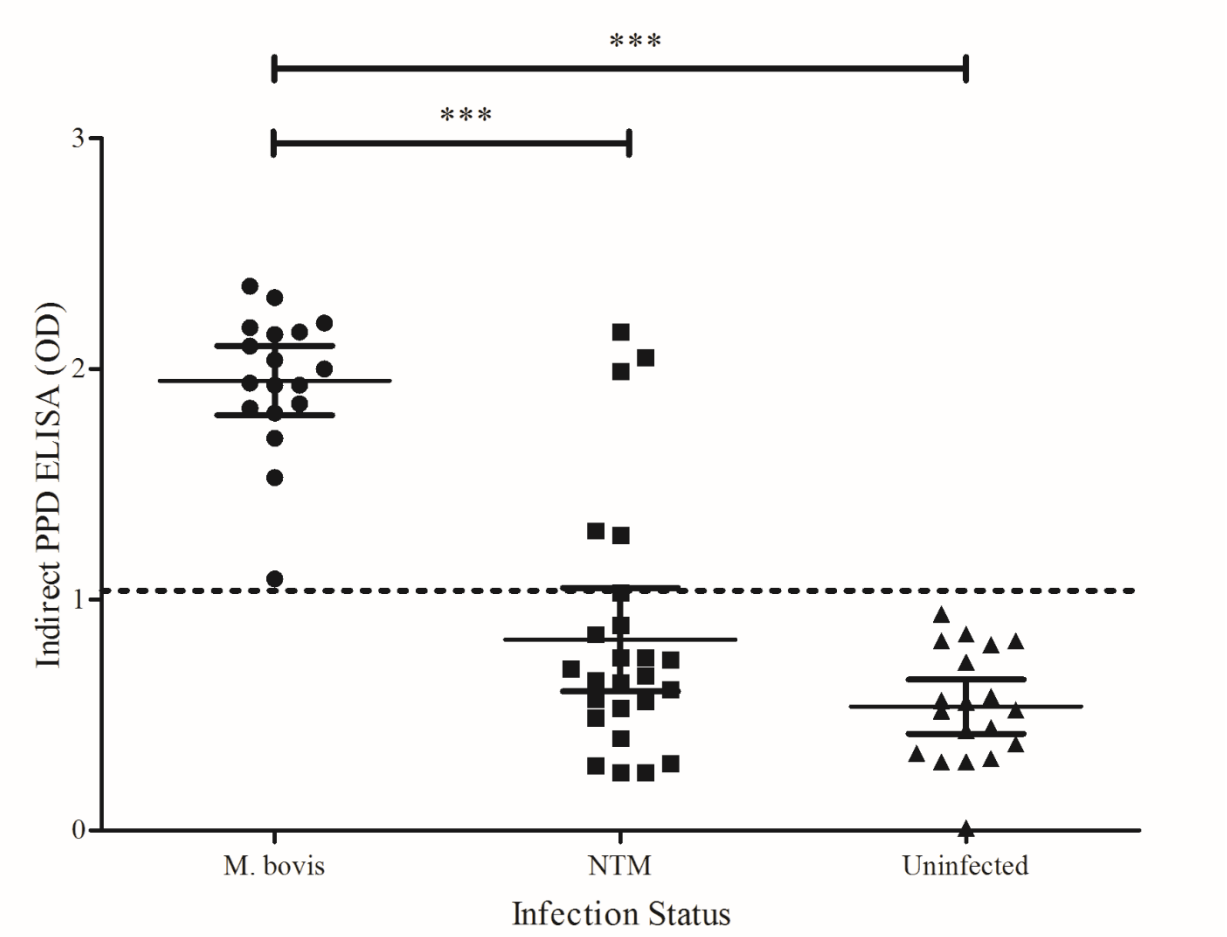


**Figure 3.1** Indirect PPD ELISA optical density (OD) values for *M. bovis* culture-positive, *M. bovis*-negative/NTM-positive (NTM), and uninfected warthogs. The dashed line indicates the cut-off value of 1.04. Mean OD and 95% CI are indicated by the horizontal bars. *** indicates $p<0.001$.

Further analyses were done by using the indirect PPD ELISA to investigate the differences in seroprevalence based on location, age and sex (Table 3.2). The multivariable logistic regression analysis conducted (controlling for all animal characteristics in the model to adjust results for potential confounding), indicated that there was a significant difference in the risk of a positive test results when comparing the four locations. Warthogs from MP were at

greater risk of being seropositive than warthogs from the other populations (Table 3.3, Fig. 3.2 and 3.3).

**Table 3.2** Seroprevalence of warthogs when using the indirect PPD ELISA based on location, age and sex with 95% confidence intervals (95% CI).

| **Location** | **Seroprevalence %** | **95% CI** |
|---|---|---|
| Marloth Park | 63 (15/24) † | 43-79 |
| uMhkuze | 31 (11/36) | 18-47 |
| Skukuza | 38 (25/91) | 26-48 |
| Satara | 16 (3/19) | 6-38 |
| Total | 38 (64/170) | 31-45 |
| **Age** | | |
| Adult | 46 (35/76) | 35-57 |
| Sub-Adult | 24 (9/37) | 13-40 |
| Juvenile | 25 (11/44) | 15-39 |
| Total | 35 (55/157) | 28-43 |
| **Sex** | | |
| Male | 37 (26/71) | 26-48 |
| Female | 34 (29/85) | 25-44 |
| Total | 35 (55/156) | 28-43 |

† Positive proportion

**Table 3.3** Risk of *M. bovis* infection as an odds ratio (OR), comparing Marloth Park warthogs with warthogs from uMhkuze, Skukuza and Satara.

| **Location** | **OR** | **P-value** |
|---|---|---|
| uMhkuze | 0.27 | 0.033 |
| Skukuza | 0.39 | 0.063 |
| Satara | 0.08 | 0.001 |

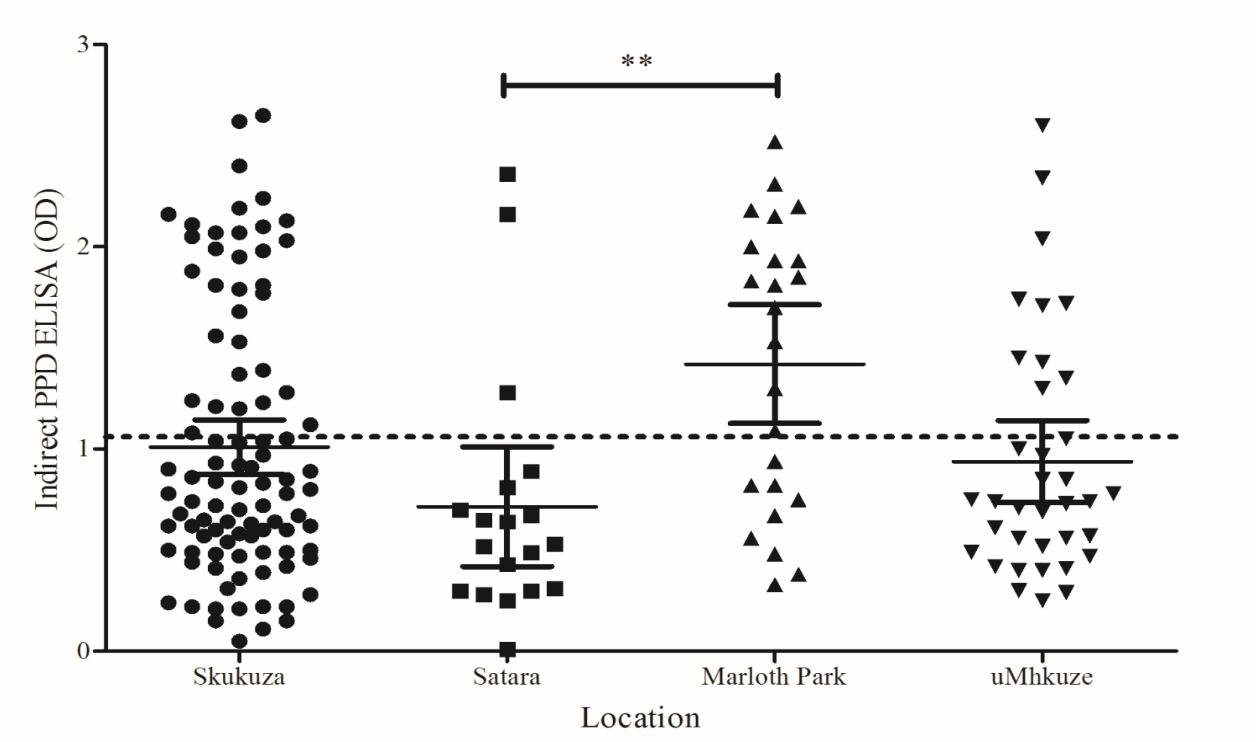


**Figure 3.2** Indirect PPD ELISA optical density (OD) values for warthogs sampled at four locations. The dashed line indicates the cut-off value set at 1.04. Mean OD and 95% CI are indicated by the horizontal bars. ** indicates $p<0.01$.

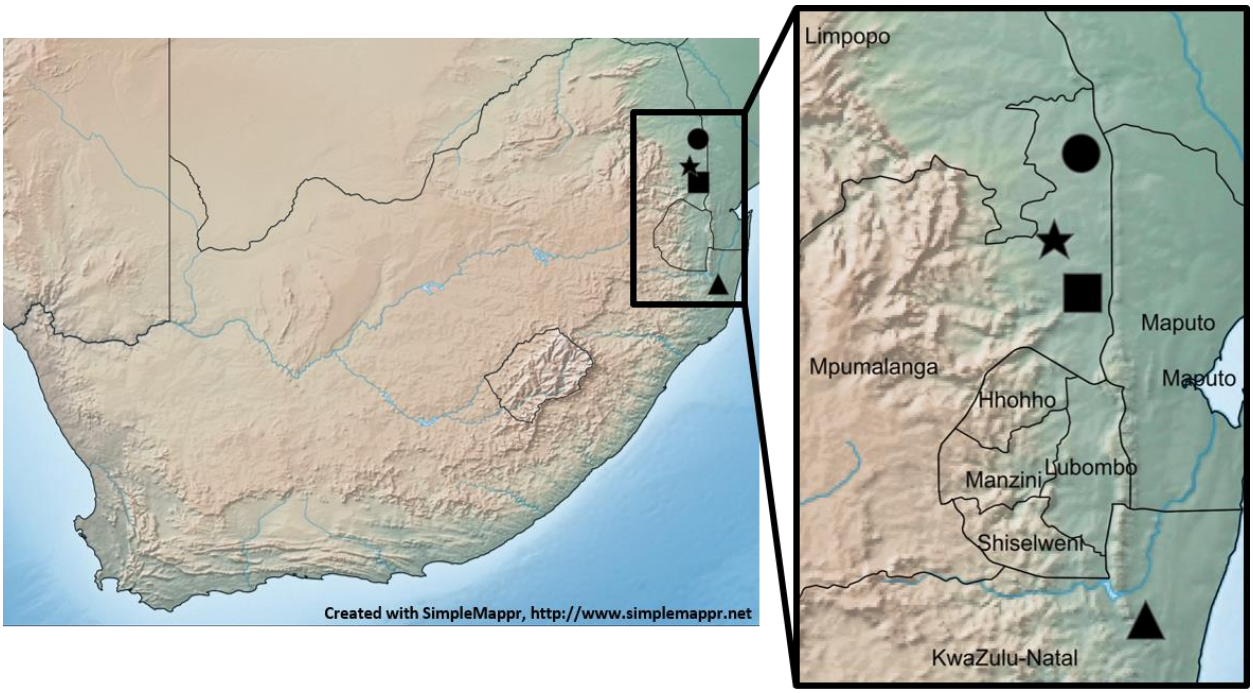


**Figure 3.3** Geographic locations of Satara (●), Skukuza (★), Marloth Park (■) and uMhkuze (▲).

Furthermore, animals sampled in SK were at lower risk of a positive result when compared to MP, but this difference was only borderline significant (Table 3.3). Sub-adult warthogs were at a lower risk of *M. bovis* infection than adults, however this difference was only borderline significant (OR=0.48, p=0.085), whereas juveniles had a significantly lower risk compared to adults (OR 0.25, p value = 0.002). Additionally, adults had a significantly higher seroreactivity than sub-adults and juveniles (Fig. 3.4). Yet, there was no apparent difference in the risk of testing positive between male and female warthogs (p=0.69) (Table 3.2).

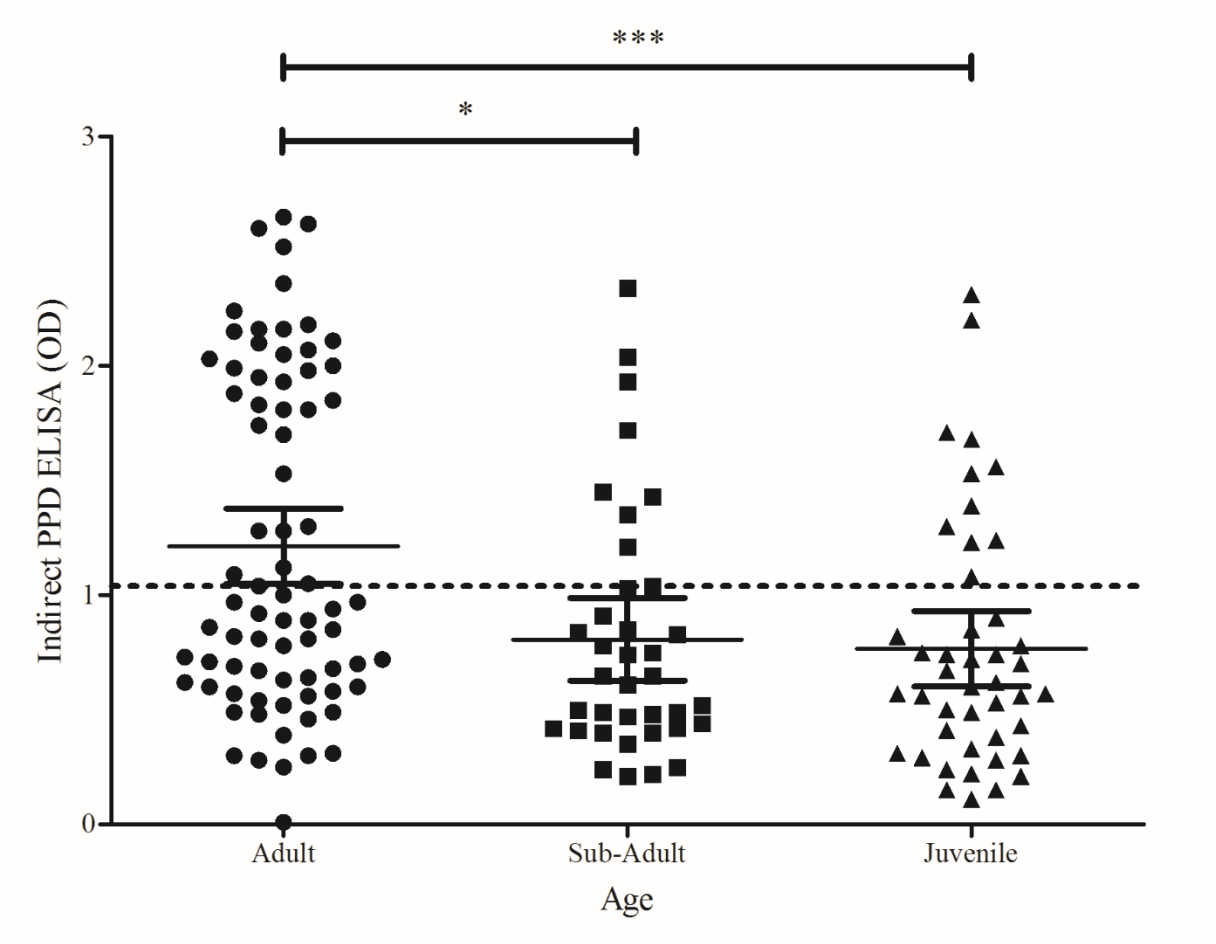


**Figure 3.4** Indirect PPD ELISA optical density (OD) values for warthogs of different age categories. The dashed line indicates the cut-off value set at 1.04. Mean OD and 95% CI are indicated by the horizontal bars. * indicates p<0.05 and *** indicates p<0.001.

## Discussion

This is the first study that has investigated *M. bovis* infection prevalence in free-ranging warthogs in uMhkuze Nature Reserve and the southern region of Greater Kruger National Park in South Africa. Using the indirect PPD ELISA, the overall seroprevalence was 38%, with 63% seroprevalence in MP, 38% in SK, 31% in MK, and 16% in SA. The overall seroprevalence was similar to the prevalence based on *M. bovis* culture results in this study (37%). The relatively high prevalence of *M. bovis* infection in warthogs in bTB endemic areas in South Africa was significantly greater than expected, since our thinking was influenced by limited studies of prevalence in this species in Uganda's Ruwenzori National Park (2%) (Woodford 1982).

Serological assays have been shown to be an accurate tool for assessing bTB prevalence in European wild boar (Aurtenetxe et al. 2008; Boadella et al. 2011; Che' Amat et al. 2015). Our results demonstrate that the indirect PPD ELISA is a useful tool to estimate prevalence and evaluate epidemiologic risk factors for *M. bovis* infection in warthogs in Africa. Two assays, the indirect PPD ELISA and TB-ELISA VK® have been previously evaluated for the detection of *M. bovis* infection in warthogs (Roos et al. 2016). In this study, these assays showed similar test performance using samples from warthogs with known culture status. The indirect PPD ELISA was chosen as an alternative to the TB-ELISA VK® for screening warthog populations since the reagents are readily available in South Africa. Developing an ELISA assay that measures a humoral response will allow retrospective studies as well as prospective studies to be done and facilitate monitoring of bTB in warthogs. The indirect ELISA assay can be used to screen warthogs from bTB endemic areas, using them as

sentinels for the presence of bTB (Nugent et al. 2002). Furthermore, the indirect ELISA's reagents are readily available to laboratories in Africa.

Since suids are commonly infected with NTMs, which may result in lesions that can be confused with bTB, it is important to determine whether serological results are influenced by these infections (Di Marco et al. 2012). In the present study, the indirect PPD ELISA could differentiate warthogs with *M. bovis* infection from those that were *M. bovis* uninfected but infected with NTMs. Therefore, seropositive results correctly identify *M. bovis*-infected animals and further support the use of serology in suids as an appropriate method to determine prevalence (Aurtenetxe et al. 2008; Boadella et al. 2011; Roos et al. 2016). However, a number of the NTM-infected warthogs (23%) had serological responses higher than the cut-off value. This could be explained by false negative *M. bovis* culture results from truly infected warthogs, which is possible since NTMs are fast-growing bacteria and potentially out compete *M. bovis* during the culture process (de la Rua-Domenech 2006; Müller et al. 2011). Another possibility is that the NTM-infected warthogs developed antibodies that recognized cross-reactive antigens in the bovine PPD coated wells. This is a potential limitation of using these assays as they do not include wells with PPD from *M. avium* or another NTM to provide a control for this seroreactivity (Aurtenetxe et al. 2008). Due to the opportunistic nature of our samples, we were not able to evaluate seroreactivity of warthogs from a *M. bovis* unexposed population in this study. Thus, to further validate the use of the indirect PPD ELISA as a diagnostic assay, future studies would need to include warthogs from *M. bovis*-unexposed populations.

Based on the high sensitivity of the indirect PPD ELISA in warthogs, a serosurvey was performed to estimate the prevalence of *M. bovis* infection in different populations in South Africa (Roos et al. 2016). Samples were available from four locations which had reported

bTB in other species. Warthogs from MP had the highest seroprevalence at 63%. Although the risk of infection for SK warthogs appeared to be lower than those in MP, this difference was not significant. Warthogs from SA and MK had significantly lower risk of infection compared to MP. These findings suggest that further investigations are needed to focus on what factors drive risk of infection in warthogs.

Previous research has shown that high host population densities and aggregation are associated with high bTB prevalence in ungulates (Vicente et al. 2007; López-Olvera et al. 2009). These could be important factors in determining whether warthogs might serve as spill-over or maintenance hosts (Renwick et al. 2007; De Garine-Wichatitsky et al. 2013). Other suids (i.e. wild boar and feral pigs) have been identified as maintenance hosts in Europe and New Zealand when animal densities are high (Di Marco et al. 2012; Nugent Gortazar and Knowles 2015). In contrast, suid populations at lower densities are considered to be spill over hosts (Santos et al. 2009; Muñoz-Mendoza et al. 2013). Furthermore, the aggregation of wild boar at watering holes has shown to increases the risk of bTB in this species (Vicente et al. 2007). Therefore, warthog density and their gregarious behaviour could potentially explain the seroprevalence differences at the study sites, however, information on animal densities and aggregation were not available for this study. Future studies should include warthog population density and aggregation data.

Being communal burrowers or dwellers (in culverts) could also attribute to the high seroprevalence in warthogs (Michel et al. 2015). The warthogs are in close contact with one another in these burrows or dwellings, which could facilitate potential transmission between warthogs (Michel et al. 2015). The microclimates in these burrows or dwellings also favour *M. bovis* survival and could lead to aerosol transmission as lesions are commonly found in

the lungs, gastrointestinal tract and sub-mandibular lymph nodes (Michel et al. 2015; Roos et al. 2016). Again, emphasising that warthogs, at high population densities, are true maintenance hosts.

Demographic risk factors (age, sex) for seropositivity were also investigated. The *M. bovis* seroprevalence was higher in adults compared to sub-adult and juvenile warthogs which might be attributed to increased bTB mortality at early ages. Nevertheless, this observation has been reported in wild boar and may reflect the time dependence of this chronic disease (Santos et al. 2009). However, other studies in wild boar have shown the opposite outcome with juveniles or sub-adults being at higher risk or no difference in infection rates between age groups (Boadella et al. 2011; Che' Amat et al. 2015). More research is needed in warthog to investigate age and infection risk.

There was no difference in seroprevalence between male and female warthogs in the present study. However, studies on wild suids have had some conflicting findings with regard to sex as a risk factor (Santos et al. 2009; Boadella et al. 2011; Di Marco et al. 2012). Most of these studies have been done in areas where wild boar are actively hunted and this could lead to confounding results since there may be changes in population demographics.

The high prevalence of *M. bovis* infection in warthogs from several populations located in bTB endemic areas in South Africa emphasizes the importance of investigating potential wildlife disease reservoirs. Differences in location and associated environmental factors may result in changes in infection pressure. Since warthogs can easily move across fences, they may present an under-recognized threat for spill-over to other hosts. The results from this

study provide a foundation for future investigations of the role that warthogs play in maintenance and spread of *M. bovis* in South Africa.

## Conflict of interest

The authors report no conflict of interest.

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# Chapter 4

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

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**My contribution to this research article:**

Planning of project
Blood collection
Blood stimulation
Running all assays
Post mortem examinations
Tissue sample collection
Mycobacterial culturing
Speciation by PCR
Data interpretation
All statistical analyses
Writing of manuscript

**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.

**Highlights:**

- Warthogs develop a measurable *M. bovis*-specific cell-mediated immune response.
- IFN-γ could not be reliably measured in stimulated warthog blood samples.
- IP-10 as a biomarker can distinguish *M. bovis* culture-positive and negative warthogs.

Keywords: Bovine tuberculosis, interferon gamma, interferon gamma induced protein 10, *Mycobacterium bovis*, warthog, wildlife

## 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 (CMI) responses (Maas et al. 2013).

*In vitro* tests commonly used to measure CMI responses 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 6kDa (ESAT-6) and culture filtrate protein 10kDa (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.

## Materials and Methods

### *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) (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, NJ, 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).

*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. 2014b). 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.

*Whole blood stimulation and cytokine assays*

All blood samples were processed within 8 hours 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, MO, USA) at a final concentration of 10 µg/ml. All samples were incubated for 24 hours 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 optimized 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 4.1). 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.

**Table 4.1** 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<br>dAb[d]: PBB0393B-050<br>RP[e]: RP0079B-005 |
| Bovine IFN-γ | P | Kingfisher Biotech Inc., | cAb: PB0156B-100<br>dAb: PBB0267B-050<br>RP: RP0013B-005 |
| Feline IFN-γ | P | Kingfisher Biotech Inc., | cAb: PB0281F-100<br>dAb: PBB0283F-050<br>RP: RP0135F-005 |
| Swine IFN-γ | P | Kingfisher Biotech Inc., | cAb: PB0157S-100<br>dAb: PBB0269S-050<br>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)<br>Bovine IFN-γ (dAb) | M | Serotech, Bio-Rad, Oxford, UK | cAb: MCA2140<br>dAb: MCA1783B |
| Human IFN-γ | M | BD Biosciences, San Jose, CA, USA | cAb: 551221<br>dAb: 554550 |

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

*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 4.1). The optimal Ab combination was selected as the pair resulting in the greatest difference in ELISA optical density (OD) obtained for the PWM-plasma and Nil-plasma pools. A single Ab combination was tested for the IP-10 assay (Table 4.1). Further optimization of both the IFN-γ and the IP-10 assays was done by testing PWM and QFT Nil samples from another four randomly selected warthogs, in duplicate. A range of plasma dilutions was tested (1:2, 1:4, 1:8 and 1:16) with the optimal dilution chosen as that resulting in the greatest differential OD between the PWM and QFT Nil samples. After selecting the optimal sample dilution, optimal cAb and dAb concentrations were determined by checkerboard titration (0.5, 1, 2 and 4 μg/ml) as the concentrations resulting in the greatest differential OD between the PWM and QFT Nil samples.

Following optimization, plasma samples from all individual warthogs were assayed in duplicate and the OD was measured at 450nm and 630nm. The differential OD values were then converted to cytokine concentrations, using a standard curve; for the IP-10 assay, a bovine IP-10 recombinant protein (King Fisher) was used and for the IFN-γ assay, a swine IFN-γ recombinant protein (Mabtech) was used as the standard (Table 4.1). Serial dilutions of the recombinant standards were made to cover the linear range of the assays (0-1000 pg/ml). The PWM-stimulated blood samples served as positive controls for cell viability, by measuring PWM-stimulated cytokine release ([PWM] – [QFT Nil]). QFT-assay results were calculated as the cytokine concentration in plasma harvested from a TB antigen-stimulated tube minus the cytokine concentration in plasma harvested from a Nil incubated tube ([QFT TB] – [QFT Nil]).

*Data analysis*

Data were captured using Manta PC Software (Dazdaq Ltd, Brighton, England) and exported to a Microsoft Excel (2013) worksheet. The data were then analysed in GraphPad Prism version 5 (GraphPad Software, March 2007). A Mann-Whitney U test was used to determine if there was a significant difference between the IP-10 QFT-assay results of *M. bovis*-infected and *M. bovis*-culture negative warthogs. A Kruskal-Wallis with Dunn's Multiple Comparison Test was performed to determine if there was a significant difference in the IP-10 QFT-assay results of *M. bovis*-infected, NTM-infected and *M. bovis*-culture negative warthogs. A receiver operator characteristic (ROC) curve analysis was performed to determine the cut-off value for the IP-10 QFT-assay, based on the Youden's index (Youden 1950). To determine if there was a significant difference between the proportion of culture-positive reults from lymph nodes with VL or NVL, the test statistic was calculated. For all analyses, a p-value $< 0.05$ was considered statistically significant.

**Results**

Of the 68 warthogs on which necropsies were performed, 19 were culture-positive for *M. bovis*. NTM were isolated from 26/68 of the warthogs and 1/68 had a co-infection of *M. bovis* and NTM (Tables 4.2 and 4.3). All VL were found in the head lymph nodes of warthogs, predominantly the sub-mandibular lymph nodes. Of the 27 warthogs that had VL at necropsy, 18 were culture-positive for *M. bovis*. Only one of the 40 warthogs with NVL had *M. bovis* isolated from tissue cultures (Tables 4.2 and 4.3). Thus, significantly more warthogs that had VL in lymph nodes had positive cultures for *M. bovis* compared to those with NVL ($z = 5.12$, $p < 0.001$). Unfortunately, in one case the detailed pathological data were not captured.

A sandwich ELISA was used to test combinations of commercially available Abs targeting IFN-γ (Table 4.1) in plasma from PWM-stimulated warthog WB and the QFT Nil plasma. The combination of Abs and plasma dilution that provided the greatest differential OD between the PWM and QFT Nil plasma was the Mabtech Swine cAb (2 µg/ml) and the Serotech Bovine dAb (0.5 µg/ml) with a plasma dilution of 1:4 (Table 4.1). All other combinations resulted in low and inconsistent OD values. Using these Abs in the assay, the PWM, QFT TB and QFT Nil plasma IFN-γ concentrations of 88 warthogs ranged from 0-4122 pg/ml (*Mdn* = 905 pg/ml; IQR 317-1575 pg/ml), 0-1279 pg/ml (*Mdn* = 24 pg/ml; IQR 0-468 pg/ml) and 0-1194 pg/ml (*Mdn* = 0 pg/ml; IQR 0-99 pg/ml), respectively. However, ELISA signals were inconsistent with expected results and provided no confidence that these were true measures of IFN-γ. No further analyses were performed to investigate the development of an IFN-γ ELISA, owing to limited plasma sample volume.

The optimised bovine IP-10 sandwich ELISA was done using the bovine recombinant IP-10 standard and a sample plasma dilution of 1:4 with 2 µg/ml cAb and 0.5 µg/ml dAb (Table 4.1). PWM-stimulated warthog plasma contained high concentrations of IP-10 (*Mdn* = 2680 pg/ml; IQR 1745-4136 pg/ml). In addition, the antigen-specific release of IP-10 was significantly greater for *M. bovis*-infected warthogs (*Mdn* = 5250 pg/ml; IQR 307-10410 pg/ml) compared to *M. bovis*-culture negative animals (*Mdn* = 129 pg/ml; IQR 33-427 pg/ml) ($p < 0.001$) (Fig. 4.1).

The IP-10 QFT-assay cut-off value was calculated using ROC curve analysis as 560 pg/ml (Se 68%, 95% CI 46-85%; Sp 84%, 95% CI 71-91%; AUC = 0.82, 95% CI 0.70-0.94). Using this cut-off value in the studied population, the majority of IP-10 positive animals were *M.*

*bovis*-infected (13/21), while most of the IP-10 negative animals were *M. bovis*-culture negative (41/47).

The IP-10 results were not affected by the presence of NTM in *M. bovis*-culture negative warthogs. The antigen-specific IP-10 concentrations for *M. bovis*-infected animals were significantly higher than for either NTM-infected (*Mdn* = 191 pg/ml; IQR 43-436 pg/ml) ($p < 0.01$) or *M. bovis*-culture negative warthogs (*Mdn* = 97 pg/ml; IQR 24-425 pg/ml) ($p < 0.001$), with no significant difference between the median values of the latter two groups (Fig. 4.1). Using the assay-specific cut-off value, a similar proportion of warthogs were IP-10 positive in the NTM-infected group (4/26) and *M. bovis*-culture negative group (4/23) ($p > 0.05$).

**Table 4.2** Summary of results for all *M. bovis*-infected warthogs. IP-10 assay results were determined using the calculated cut-off value (560 pg/ml). All cultures underwent speciation using the 16S DNA sequencing and a RD-analysis to confirm *M. bovis* presence.

| Animal ID | IP-10 (pg/ml) | IP-10 assay result | Culture result | Pathology |
|---|---|---|---|---|
| 15/248 | 6042,4 | Positive | *M. bovis* | VL[a] |
| 15/249 | 13454,4 | Positive | *M. bovis* | VL |
| 15/250 | 10063,2 | Positive | *M. bovis* | VL |
| 15/251 | 5249,6 | Positive | *M. bovis* | VL |
| 15/264 | 10111,0 | Positive | *M. bovis* | VL |
| 15/265 | 39068,9 | Positive | *M. bovis* | VL |
| 15/266 | 12623,7 | Positive | *M. bovis* | VL |
| 15/267 | 10407,5 | Positive | *M. bovis* | VL |
| 15/268 | 581,5 | Positive | *M. bovis* | VL |
| 15/270 | 8094,5 | Positive | *M. bovis* | VL |
| 15/306 | 1004,9 | Positive | *M. bovis* | VL |
| 15/513 | 828,3 | Positive | *M. bovis* | VL |
| 15/140 | 39988,2 | Positive | *M. bovis*, *M. intracellulare* | VL |
| 15/263 | 339,3 | Negative | *M. bovis* | VL |
| 15/269 | 160,9 | Negative | *M. bovis* | VL |
| 15/300 | 150,2 | Negative | *M. bovis* | VL |
| 15/W/004 | 187,9 | Negative | *M. bovis* | NVL[b] |
| 15/W/008 | 0,0 | Negative | *M. bovis* | VL |
| 15/W/018 | 307,1 | Negative | *M. bovis* | VL |

[a]Visible bTB-like lesion
[b]No visible bTB-like lesion

**Table 4.3** Summary of results for all NTM-infected and culture-negative warthogs. IP-10 assay results were derived from the calculated cut-off value (560 pg/ml). All speciation was done using 16S DNA sequencing.

| Animal ID | IP-10 (pg/ml) | IP-10 assay result | Culture result | Pathology |
|---|---|---|---|---|
| 15/305 | 1280,7 | Positive | *M. asiaticum* | NVL[a] |
| 15/516 | 219,4 | Negative | *M. asiaticum* | NVL |
| 15/309 | 42,9 | Negative | *M. avium* | NVL |
| 15/W/005 | 263,0 | Negative | *M. avium* | VL[b] |
| 15/W/006 | 115,8 | Negative | *M. avium* | NVL |
| 15/W/015 | 457,8 | Negative | *M. avium* | VL |
| 15/W/020 | 94,3 | Negative | *M. avium* | NVL |
| 14/227 | 5854,2 | Positive | *M. intracellulare* | ND |
| 14/335 | 428,2 | Negative | *M. intracellulare* | NVL |
| 15/W/007 | 0,0 | Negative | *M. intracellulare* | VL |
| 15/301 | 664,0 | Positive | *M. intracellulare* | NVL |
| 15/535 | 419,9 | Negative | *M. paraffinicum* | NVL |
| 15/W/002 | 43,6 | Negative | *M. parascrofulaceam* | VL |
| 15/W/019 | 281,0 | Negative | *M. parascrofulaceam* | VL |
| 15/816 | 0,0 | Negative | *M. scrofulaceum* | NVL |
| 15/W/012 | 1082,6 | Positive | *M. scrofulaceum* | NVL |
| 15/514 | 530,8 | Negative | *M. simiae* | NVL |
| 15/517 | 66,0 | Negative | *M. simiae* | NVL |
| 15/534 | 0,0 | Negative | *M. simiae* | NVL |
| 15/536 | 128,7 | Negative | *M. simiae* | NVL |
| 15/758 | 0,0 | Negative | *M. simiae* | NVL |
| 15/759 | 290,5 | Negative | *M. simiae* | NVL |
| 15/788 | 0,0 | Negative | *M. simiae* | NVL |
| 15/815 | 126,1 | Negative | *M. simiae* | NVL |
| 15/W/011 | 203,6 | Negative | *M. simiae* | NVL |
| 15/515 | 178,6 | Negative | *M. yongonense* | NVL |
| 15/137 | 70,2 | Negative | *M. bovis*-negative | NVL |
| 15/138 | 895,1 | Positive | *M. bovis*-negative | NVL |
| 15/262 | 537,4 | Negative | *M. bovis*-negative | VL |
| 15/271 | 425,2 | Negative | *M. bovis*-negative | VL |
| 15/302 | 52,5 | Negative | *M. bovis*-negative | NVL |
| 15/304 | 693,2 | Positive | *M. bovis*-negative | NVL |
| 15/307 | 2025,0 | Positive | *M. bovis*-negative | NVL |
| 15/308 | 53,1 | Negative | *M. bovis*-negative | NVL |
| 15/310 | 912,6 | Positive | *M. bovis*-negative | NVL |
| 15/760 | 380,4 | Negative | *M. bovis*-negative | NVL |
| 15/789 | 30,0 | Negative | *M. bovis*-negative | NVL |
| 15/790 | 36,5 | Negative | *M. bovis*-negative | NVL |
| 15/791 | 265,6 | Negative | *M. bovis*-negative | NVL |
| 15/792 | 284,6 | Negative | *M. bovis*-negative | NVL |
| 15/814 | 99,3 | Negative | *M. bovis*-negative | NVL |
| 15/W/003 | 423,2 | Negative | *M. bovis*-negative | NVL |
| 15/W/009 | 0,0 | Negative | *M. bovis*-negative | NVL |
| 15/W/010 | 3,4 | Negative | *M. bovis*-negative | NVL |
| 15/W/013 | 23,9 | Negative | *M. bovis*-negative | VL |
| 15/W/016 | 0,0 | Negative | *M. bovis*-negative | NVL |
| 15/W/017 | 0,0 | Negative | *M. bovis*-negative | NVL |
| 15/W/021 | 97,2 | Negative | *M. bovis*-negative | NVL |
| 15/W/025 | 0,0 | Negative | *M. bovis*-negative | VL |

[a]No visible bTB-like lesion
[b]Visible bTB-like lesion

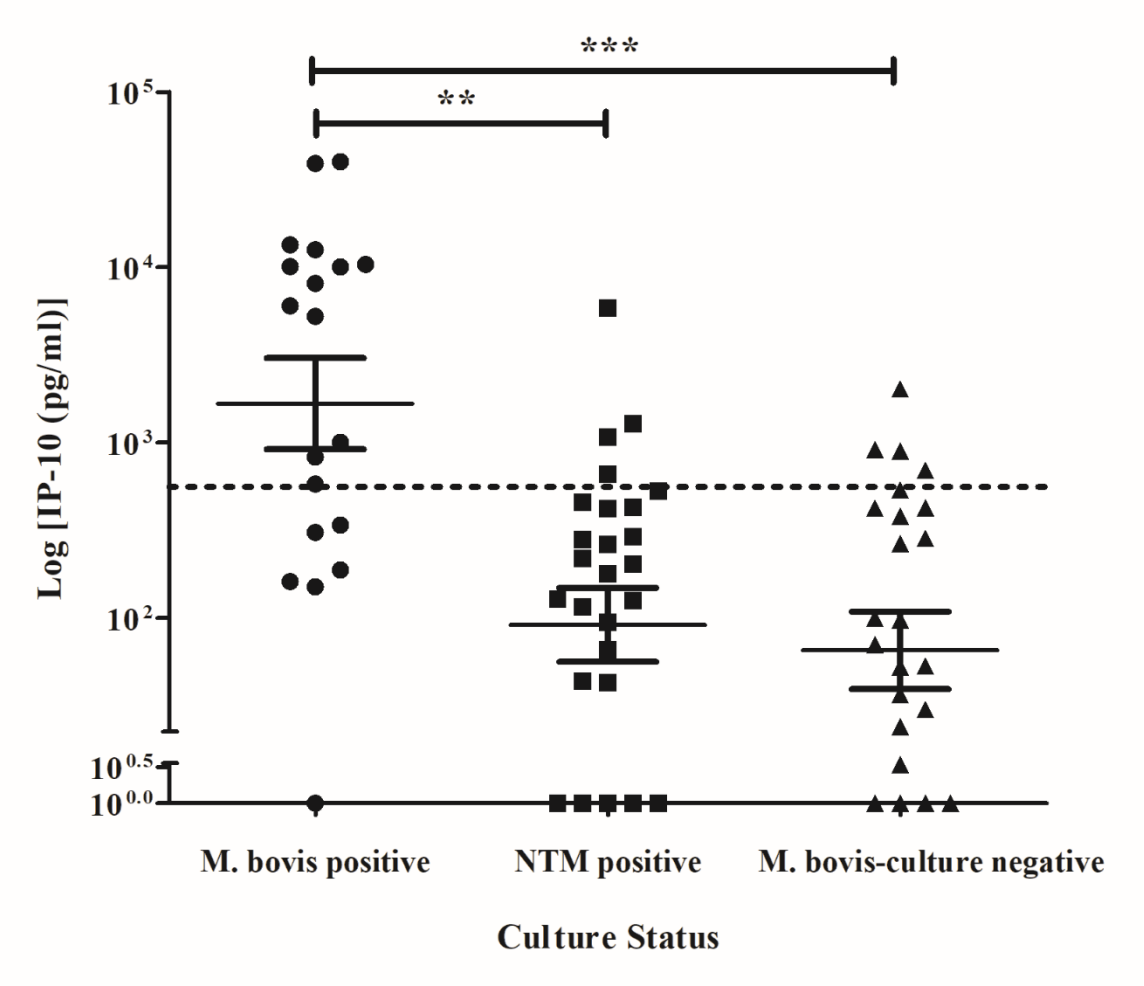


**Figure 4.1** Concentration of antigen-specific IP-10 in QFT stimulated whole blood from warthogs with *M. bovis*-culture positive, NTM-culture positive and those that were *M. bovis*-culture negative. Median and interquartile ranges are shown by the bars. The dotted line is the warthog-specific cut-off value calculated from the ROC curve analysis (560 pg/ml). Data on y-axis was transformed using a log scale. ** indicate $p<0.01$; *** indicate $p<0.001$

Overall, 37 of the 88 *M. bovis*-exposed warthogs were IP-10 test-positive. The test positive samples had high levels of IP-10 with a range from 582-39988 pg/ml (*Mdn* = 3035 pg/ml; IQR 1162-10087 pg/ml) (Fig .4.1).

## Discussion

*In vitro* assays of CMI responses use diagnostic biomarkers to detect *M. bovis* infection. Since CMI responses have not been described in warthogs, the aim of this study was to develop and evaluate cytokine release assays, by measuring IFN-γ and IP-10 in QFT-stimulated WB, for the detection of *M. bovis* infection in this species. Although results of the IFN-γ ELISA were inconsistent, the bovine IP-10 ELISA was able to detect this cytokine in mitogen-stimulated warthog WB. In addition, antigen-specific IP-10 could distinguish *M. bovis*-infected warthogs from *M. bovis*-culture negative animals in a *M. bovis*-exposed population. An assay-specific cut-off value was determined as 560 pg/ml, resulting in an assay Se of 68%. Therefore, IP-10 appears to be a promising biomarker of antigen-specific immune responses in warthogs and warrants further investigation.

Initial investigation of cytokine release assays in this study included IFN-γ since its use as a biomarker of TB is well established and has been studied in a variety of species, from humans to domestic animals and wildlife (Rothel et al. 1990; Pai et al. 2004; Maas et al. 2013). Although an IGRA has been reported for pigs (Pesciaroli et al. 2012), the results of the IFN-γ QFT assay in warthogs were unreliable, owing to numerous issues such as high background in nil samples, and generally weak signal in antigen-stimulated samples. One possible explanation is that the Abs used against IFN-γ in the current study were not able to detect warthog IFN-γ, despite reports of broad cross-reactivity towards suid cytokines by the manufacturers. Other causes may be due to poor IFN-γ release in response to the antigen-specific peptides, or the presence of inhibitors or nonspecific substances in the warthog plasma that interfered in the ELISA. Difficulty detecting IFN-γ in stimulated blood samples has been observed in other species, e.g. lions (S. Parsons pers. comm. 2017). Since there were

limited volumes of plasma and inconsistent detection, further development of the IGRA was not pursued.

In addition to IFN-γ, other cytokine biomarkers have been used in blood-based assays to identify *M. bovis* infection in a number of animal species (Goosen et al. 2014a,b; Ruhwald et al. 2009). Low levels of IFN-γ in antigen-specific stimulated WB have been shown to induce high levels of IP-10, making IP-10 a more sensitive marker of TB than IFN-γ in humans and buffaloes (Ruhwald et al. 2008; Goosen et al. 2015). Since the Kingfisher anti-bovine IP-10 Abs are broadly cross-reactive with other species (including swine), this assay was evaluated for detection of warthog IP-10 (Kingfisher 2018).

The bovine IP-10 ELISA could detect warthog IP-10 in plasma harvested from antigen-stimulated whole blood and differentiate between *M. bovis*-infected and *M. bovis*-culture negative warthogs. The assay-specific IP-10 cut-off value of 560 pg/ml is not a diagnostic cut-off but rather used for the study population since these warthogs were from bTB endemic areas and considered potentially exposed. It should also be noted that antigen specific release of warthog IP-10 (*Mdn* = 5250 pg/ml) is similar to that of African buffaloes using the QFT stimulation platform (*Mdn* = 5458pg/ml) (Goosen et al. 2015).

The warthog IP-10 assay was not as sensitive (68%) as expected. This might be due to differences in IP-10 between bovids and suids, resulting in a lower antibody cross-reactivity in warthogs than predicted for swine. Although the bovine IP-10 ELISA could detect warthog IP-10, the use of swine-specific antibodies may improve diagnostic performance. Another important aspect affecting evaluation of test performance is the sensitivity of mycobacterial culture. This gold standard used in this study may not be as sensitive as *in vitro* assays for

detection of infection (de la Rua-Domenech et al. 2006). Lack of detection of infection by culture may explain the IP-10 positive warthogs among the *M. bovis*-culture negative group. Therefore, the IP-10 QFT assay Se might be underestimated in our study.

The presence of VL in head lymph nodes was indicative of *M. bovis* infection in this exposed population, which is similar to observations in other suids, and there was a good correlation with IP-10 results (Santos et al. 2010; Pesciaroli et al. 2012). However, seven warthogs with NVLs had positive IP-10 QFT assay results, suggesting that these were also infected, although only one had a positive *M. bovis* culture result. The lack of culture sensitivity may miss truly infected animals especially in early stages of infection when there are no visible lesions. Further studies are needed to determine the sensitivity of the IP-10 QFT assay in different stages of infection.

*M. bovis*-infected warthogs had a significantly greater IP-10 response than those from which NTM were isolated, with no difference in IP-10 between the NTM-infected and *M. bovis*-culture negative warthogs. Therefore, although the warthogs originated from a *M. bovis*-exposed population, the low proportion (8/49) of IP-10 positive warthogs in the *M. bovis*-culture negative cohort suggests that the assay is specific for *M. bovis*.

The overall IP-10 prevalence of 42% in the 88 warthogs screened closely reflected our expected infection prevalence in this *M. bovis*-exposed warthog population, based on tissue culture (37%) and serological results (38%) from other studies (Roos et al. 2018). One of the limitations of this study was that samples were only available from warthogs in bTB endemic regions, due to opportunistic sampling. Further evaluation of the IP-10 assay will require

obtaining samples from an unexposed warthog population to determine a diagnostic cut-off value and assay specificity.

The development of a multi-species IP-10 assay would be a useful tool for detecting *M. bovis* infection in wildlife populations and facilitate screening in areas with bTB. Although only one set of IP-10 capture and detection antibodies was evaluated using warthog samples, this assay has been previously shown to be useful for African buffaloes (Goosen et al. 2015). Therefore, IP-10 should be further investigated as a bTB biomarker in other wildlife species.

In summary, we had inconsistent results for the IFN-γ assay. However, we were able to detect the release of IP-10 in QFT-stimulated WB from warthogs. The use of an IP-10 assay-specific cut-off value resulted in the accurate detection of *M. bovis* infected warthogs, with no difference between the QFT-assay results of NTM-infected and *M. bovis*-culture negative warthogs. Thus, IP-10 could be a potential diagnostic biomarker in this species.

**Conflict of interest**

The authors report no conflict of interest.

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# Chapter 5

## Detection of *Mycobacterium bovis*-infected warthogs (*Phacochoerus africanus*) using the intradermal tuberculin test

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**My contribution to this research article:**

Planning of project
Collecting data
Post mortem examinations
Tissue sample collection
Mycobacterial culturing
Speciation by PCR
Data interpretation
All statistical analyses
Writing of manuscript

**Abstract**

Bovine tuberculosis (bTB) caused by *Mycobacterium bovis* has previously been diagnosed in warthogs and infection can be highly prevalent (> 30%) in endemic areas. Thus, warthogs could potentially be an important species to consider as sentinels for disease surveillance. However, disease surveillance is dependent on availability of accurate diagnostic assays and only a few diagnostic tests have been investigated for warthogs. Furthermore, the tests that have been used in this species require laboratory equipment and trained personnel to obtain results. Therefore, this study investigated the use of the intradermal tuberculin test (ITT) to screen warthogs for bTB, which can be done with minimal equipment and under field conditions by most veterinarians and other qualified professionals. Changes in skin fold thickness measurements at the bovine purified protein derivative (PPD) administration site, between 0 and 72 hours, were compared with differential changes between the bovine and avian PPD sites, for 34 warthogs, to evaluate the performance when different interpretation criteria for the ITT was used. Using an increase of 1.8 mm or more at the bovine PPD site as a cut-off for positive responders, 69% of 16 *M. bovis* culture-positive warthogs had a positive test result, with 100% of the 18 culture-negative warthogs considered as test negative. When a differential of 1.2 mm or more in skin fold thickness at the bovine PPD compared to the avian PPD site was used as a cut-off for the comparative ITT, 81% of culture-positive warthogs were considered as test positive, with 100% of culture-negative warthogs considered as test negative. The findings in this study suggest that the ITT is a promising tool to use when screening warthogs for *M. bovis* infection.

**Highlights:**

- The tuberculin skin test can be used to identify *M. bovis*-infected and uninfected warthogs.

- The comparative intradermal tuberculin test is more sensitive than the single intradermal tuberculin test.

Keywords: Bovine tuberculosis, screening test, *Mycobacterium bovis*, intradermal tuberculin test, warthog, wildlife

## Introduction

The primary cause of bovine tuberculosis (bTB) is an acid-fast bacterium, *Mycobacterium bovis*, which has been reported to infect more than 17 wildlife species in South Africa (Michel et al. 2006). The disease has become endemic in some nature reserves and private game farms within South Africa (Hlokwe et al. 2014). Furthermore, cases of bTB have been reported in threatened or endangered species, such as lions (*Panthera leo*) and rhinoceros (*Ceratotherium simum* and *Diceros bicornis*) (Miller et al. 2017; Sylvester et al. 2017). Certain wildlife species have become maintenance hosts of the disease in South Africa including the African buffalo (*Syncerus caffer*) (De Vos et al. 2001). Warthogs are also known to become infected with *M. bovis* and could potentially act as a maintenance host in endemic areas (Renwick et al. 2007; Roos et al. 2016). This species is capable of crossing fences and other man-made barriers, which could lead to dissemination of disease, as is the case for wild boar (*Sus scrofa*), a bTB maintenance host in the Iberian Mediterranean ecosystem (Michel et al. 2006; Naranjo et al. 2008). Moreover, similar to feral pigs, warthogs may serve as a good sentinel as they are highly susceptible to this infection (Roos et al. 2018).

Accurate diagnostic tests are needed for disease surveillance. However, only a limited number of assays are available for bTB diagnosis in African wildlife species. A lack of approved laboratory facilities and logistical difficulties in getting samples to laboratories from disease controlled and remote areas also limits wildlife testing. One available method for field detection of bTB is to euthanase animals, identify granulomatous lesions on necropsy, and confirm the diagnosis using mycobacterial culture. However, it can take 6-8 weeks before culture results become available. Therefore, there is a need to have an accurate field-friendly ante-mortem assay for bTB screening of species such as warthogs, which can be readily performed by veterinarians.

The Intradermal Tuberculin Test (ITT) has been used for bTB detection in a range of species including domestic cattle, wild boar, white-tailed deer (*Odocoileus virginianus*), elk (*Cervus canadensis*), African buffalo, and lions (Palmer et al. 2001, 2011; de la Rua-Domenech et al. 2006; Jaroso et al. 2010; Keet et al. 2010; Goosen et al. 2014). The ITT is readily available to veterinarians and can be performed in the field, providing a result within 72 hours. The objective of this study was to investigate the utility and test performance of the ITT for detection of *M. bovis* infection in warthogs.

## Methods

### *Animals and sampling*

In 2015, warthogs were captured and culled as part of drought management in the Greater Kruger National Park area (GKNP) by park veterinarians (SANParks 2006). Since bTB is endemic in the GKNP, all warthogs in this study were considered exposed to *M. bovis*. Sixteen female and eighteen male warthogs were first immobilized and held in quarantine bomas to evaluate performance of the ITT in this species, as described below. After reading

the ITT, immobilized warthogs were humanely euthanized, and a full necropsy performed as part of a disease surveillance program. Post-mortem examination and tissue sampling were performed as previously described (Roos et al. 2016).

*Immobilization*

All warthogs were immobilized using a drug combination of (i) zolazepam-tiletamine (Zoletil®; Virbac RSA, (Pty) Ltd, Centurion, South Africa) in combination with azaperone (Kyron Laboratories (Pty) Limited, Benrose, South Africa) or medetomidine (Kyron), or (ii) azaperone, butorphanol (Kyron), medetomidine and ketamine (Kyron) (Moon and Smith 1996; SANParks 2006; 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.

*Intradermal Tuberculin Test (ITT)*

The intradermal tuberculin test was performed as described elsewhere (Keet et al. 2010). Briefly, the skin fold thickness (SFT) caudal to each ear was measured using a spring loaded Hauptner calliper with pistol grip prior to administration of the purified protein derivative (PPD; Institute for Animal Sciences, Lelystadt, Netherlands) injection (Fig 5.1). PPD was injected intradermally at 0 h: 0.2 ml bovine PPD (30 000 IU/ml) ($PPD_b$) on the left and 0.2 ml avian PPD (25 000 IU/ml) ($PPD_a$) on the right After 72 h, the SFT at each PPD injection site was measured and examined for signs of oedema, heat, exudation or necrosis (OIE 2009). The same experienced operator performed all measurements and PPD injections.


**Figure 5.1** Purified protein derivative injection site for the intradermal tuberculin test, caudal to each ear. The picture shows the appropriate use of the callipers to measure the skin fold thickness (mm).

The ITT was interpreted in two ways. For the single intradermal tuberculin test (SITT), the SFT prior to the $PPD_b$ injection (0 h) was subtracted from the measurement at the same site 72 h post-injection ($\Delta$ $PPD_b$). For the comparative intradermal tuberculin test (CITT), the 72 h SFT at the $PPD_a$ injection site was subtracted from that at the $PPD_b$ injection site ($PPD_{b-a}$). The $\Delta$ $PPD_a$ was calculated in the same way as the $\Delta$ $PPD_b$.

*Mycobacterial cultures and speciation*

All tissue samples were processed using the BACTEC™ MGIT™ 960 system (BD Biosciences, Franklin Lakes, NJ, USA) as previously described (Goosen et al. 2014). Positive cultures in the MGIT system were further analysed by Ziehl-Neelsen (ZN) staining and all ZN-positive cultures were speciated using genetic region of difference analysis (Warren et al. 2006) and 16S DNA sequencing (Leclerc et al. 2000). The status of warthogs as *M. bovis*-infected or uninfected was based on culture results.

*Data analysis*

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, March 2007). The $\Delta$ $PPD_b$ and $\Delta$ $PPD_a$ values for *M. bovis* culture-positive and culture-negative animals were compared within and between groups using a Kruskal-Wallis statistic with a Dunn's Multiple Comparison test. The $\Delta$ $PPD_a$ and $\Delta$ $PPD_b$ comparisons were done to confirm the specific response of *M. bovis* culture-positive warthogs to the $PPD_b$ injection. Evaluation of the $\Delta$ $PPD_a$ was done to provide information as to whether non-tuberculous mycobacteria (NTM) influenced the $PPD_b$ responses. The SITT results for culture-positive and culture-negative animals were compared using a Mann Whitney test, as were results for the CITT. Warthog specific cut-off values to determine positive responders were calculated using a receiver operator characteristic (ROC) curve analysis and selected based on the Youden's index (Youden 1950).

## Results

The ITT responses were measured in all 34 warthogs and individuals divided into two study cohorts based on mycobacterial culture results (Table 5.1). *M. bovis*-infection was confirmed by mycobacterial culture in 16 of the 34 warthogs. The SFT measurements from warthogs

**Table 5.1** Raw data from 34 warthogs' skinfold measurements (in mm) to $PPD_b$ and $PPD_a$ at time points 0 h and 72 h post-injection, the Δ $PPD_b$ and Δ $PPD_a$ as well as each warthogs' mycobacterial culture result.

| Lab no. | $PPD_b$ 0 hr | $PPD_b$ 72 hr | Δ $PPD_b$ | $PPD_a$ 0 hr | $PPD_a$ 72 hr | Δ $PPD_a$ | Bacterial Culture |
|---|---|---|---|---|---|---|---|
| 15/137 | 3 | 3 | 0 | 3,1 | 3,1 | 0 | Culture negative |
| 15/138 | 3,6 | 4,1 | 0,5 | 3,6 | 4,8 | 1,2 | Culture negative |
| 15/262 | 4,2 | 3,8 | -0,4 | 4,6 | 5 | 0,4 | Culture negative |
| 15/271 | 4,9 | 4,9 | 0 | 4,3 | 4,8 | 0,5 | Culture negative |
| 15/302 | 2,6 | 4 | 1,4 | 2,7 | 4,6 | 1,9 | Culture negative |
| 15/304 | 3 | 3 | 0 | 3 | 3,3 | 0,3 | Culture negative |
| 15/307 | 5 | 6,1 | 1,1 | 4 | 6,6 | 2,6 | Culture negative |
| 15/308 | 3,3 | 3,3 | 0 | 3,7 | 3,8 | 0,1 | Culture negative |
| 15/310 | 3 | 3,2 | 0,2 | 3 | 3,4 | 0,4 | Culture negative |
| 15/305 | 3,2 | 4,2 | 1 | 2,8 | 3,1 | 0,3 | *M. asiaticum* |
| 15/516 | 5,3 | 4,2 | -1,1 | 5,5 | 4,7 | -0,8 | *M. asiaticum* |
| 15/309 | 3,2 | 3,6 | 0,4 | 3,3 | 3,4 | 0,1 | *M. avium* |
| 15/301 | 2,3 | 2,4 | 0,1 | 2,6 | 2,5 | -0,1 | *M. intracellulare* |
| 15/515 | 3,9 | 3,4 | -0,5 | 3,3 | 3 | -0,3 | *M. intracellulare* |
| 15/535 | 3,8 | 3,8 | 0 | 5,5 | 5,2 | -0,3 | *M. paraffinicum* |
| 15/514 | 4,8 | 4,7 | -0,1 | 4 | 5,2 | 1,2 | *M. simiae* |
| 15/534 | 4,6 | 5,3 | 0,7 | 4,8 | 4,2 | -0,6 | *M. simiae* |
| 15/536 | 4,5 | 4,5 | 0 | 4,5 | 5 | 0,5 | *M. simiae* |
| 15/140 | 4,8 | 6,9 | 2,1 | 4,6 | 4,6 | 0 | *M. bovis* |
| 15/248 | 3,8 | 8 | 4,2 | 4 | 3,8 | -0,2 | *M. bovis* |
| 15/249 | 4,4 | 4,4 | 0 | 4,5 | 4,7 | 0,2 | *M. bovis* |
| 15/250 | 3,6 | 7 | 3,4 | 4,3 | 4,1 | -0,2 | *M. bovis* |
| 15/251 | 4,8 | 5,7 | 0,9 | 4,1 | 4 | -0,1 | *M. bovis* |
| 15/263 | 4,1 | 7,2 | 3,1 | 3,9 | 3,8 | -0,1 | *M. bovis* |
| 15/264 | 3,8 | 14 | 10,2 | 3,2 | 3,4 | 0,2 | *M. bovis* |
| 15/265 | 4,3 | 4,9 | 0,6 | 3,8 | 3,8 | 0 | *M. bovis* |
| 15/266 | 4,3 | 11,2 | 6,9 | 4,2 | 3,6 | -0,6 | *M. bovis* |
| 15/267 | 3,8 | 6,5 | 2,7 | 4,4 | 4,6 | 0,2 | *M. bovis* |
| 15/268 | 3,6 | 6,4 | 2,8 | 3,6 | 4,1 | 0,5 | *M. bovis* |
| 15/269 | 3,7 | 4,7 | 1 | 2,9 | 3,5 | 0,6 | *M. bovis* |
| 15/270 | 2,8 | 5,9 | 3,1 | 2,9 | 3,2 | 0,3 | *M. bovis* |
| 15/300 | 3,6 | 7 | 3,4 | 3,6 | 4 | 0,4 | *M. bovis* |
| 15/306 | 3,3 | 8,1 | 4,8 | 3,8 | 4,6 | 0,8 | *M. bovis* |
| 15/513 | 4,8 | 5,1 | 0,3 | 4,6 | 6,2 | 1,6 | *M. bovis* |

infected with NTMs were not significantly different from those of *M. bovis* culture-negative warthogs, for both PPD sites, and therefore grouped as culture-negative (p = 0.086). There was no significant difference in Δ $PPD_a$ measurements between *M. bovis* culture-positive and culture-negative warthogs (p = 0.650, Fig.5.2, Table 5.2).

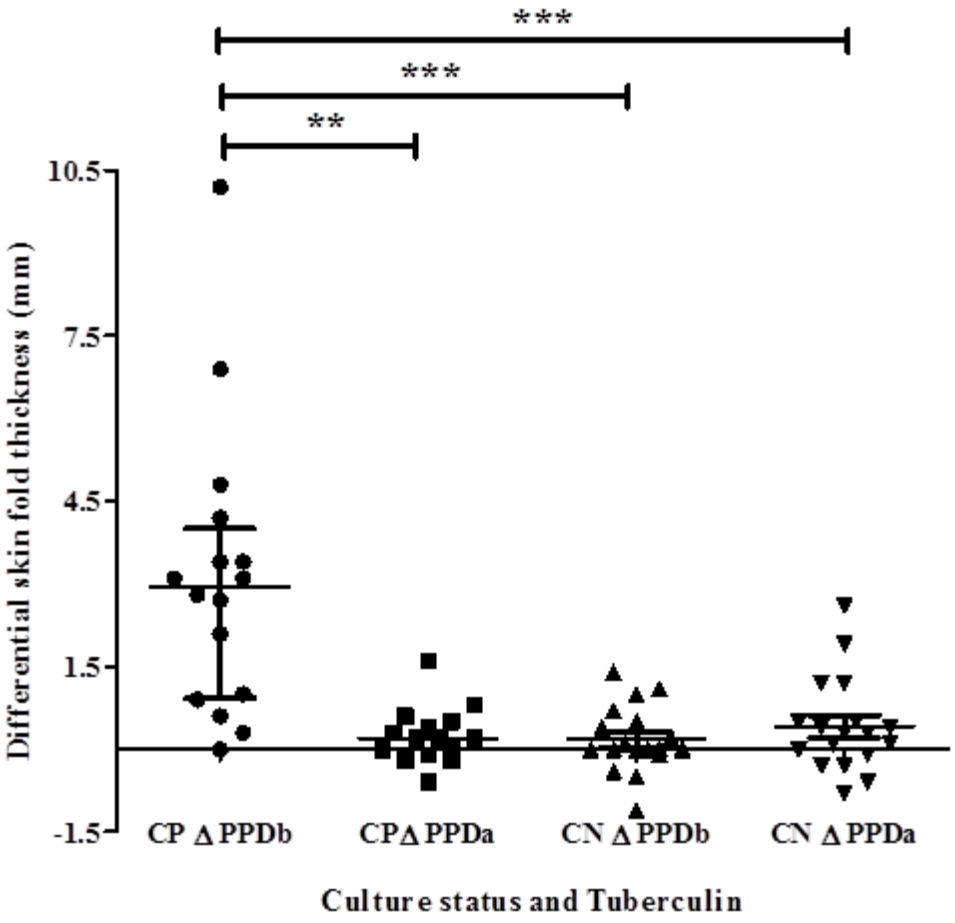


**Figure 5.2** Differential in skin fold thickness measurement at the $PPD_b$ and $PPD_a$ (Δ $PPD_b$ and Δ $PPD_a$) injection sites after 72 h in *M. bovis* culture-positive (CP) or culture-negative (CN) warthogs. Median and interquartile ranges are represented by the horizontal bars. ** indicates p<0.01, *** indicates p<0.001.

However, Δ $PPD_b$ measurements were significantly greater for *M. bovis* culture-positive compared to culture-negative animals (Fig. 5.3, Table 5.2). Furthermore, in *M. bovis* culture-positive warthogs, the increase in SFT at the $PPD_b$ site (Δ $PPD_b$) was significantly greater than at the $PPD_a$ site (Δ $PPD_a$) ($p = 0.002$), although no differences between these measurements were seen in culture-negative warthogs ($p = 0.128$, Fig. 5.2, Table 5.2). The $PPD_{b-a}$ values were significantly greater for culture-positive warthogs compared to culture-negative warthogs ($p < 0.0001$, Fig.5.4, Table 5.2).

A warthog-specific cut-off value for the SITT was calculated as ≥ 1.8mm using a ROC curve analysis (AUC = 0.91, 95% CI 0.81-1.0) (Table 5.3). Based on this cut-off, 11 out of the 16 *M. bovis* culture-positive warthogs were SITT-positive (69%), while none of the 18 culture-negative warthogs had a positive test result (100%).

The cut-off value for the CITT was ≥ 1.2mm (AUC = 0.91, 95% CI 0.79-1.0) (Table 5.4). This cut-off value resulted in 13 of 16 culture-positive warthogs being CITT-positive (81%) and classified all 18 culture-negative warthogs as test negative (100%). No signs of oedema, heat, exudation or necrosis were observed at the PPD injection sites in any of the 34 warthogs tested.

**Table 5.2** Median values of the skinfold increase (in mm) using different combinations of measurements for the ITT in *M. bovis* culture-positive and culture–negative warthogs. Interquartile ranges are shown in parentheses.

| Skinfold reading | Culture Positive | Culture Negative |
|---|---|---|
| Δ $PPD_a$ | 0.2mm (-0.1-0.5) | 0.3mm (-0.2-0.7) |
| Δ $PPD_b$ | 3.0mm (0.9-4.0) | 0.0mm (-0.1-0.6) |
| $PPD_b$ - $PPD_a$ | 2.5mm (1.3-3.5) | -0.4mm (-0.5-0.1) |

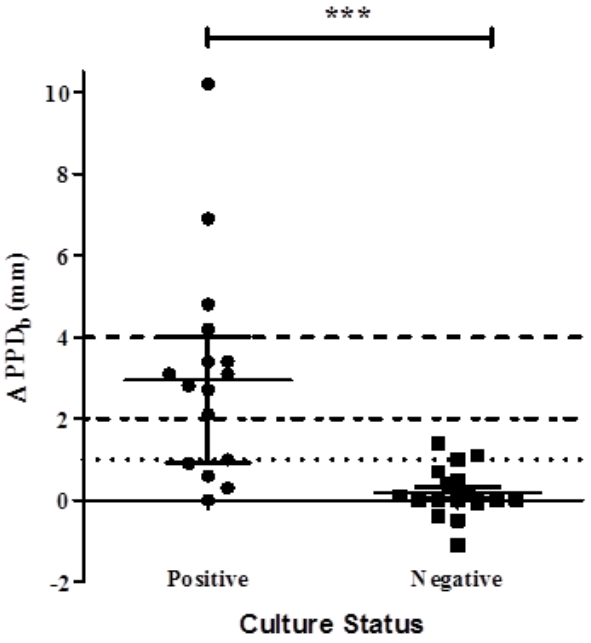


**Figure 5.4** Differential in skin fold thickness (Δ $PPD_b$) at the $PPD_b$ injection site after 72 h in *M. bovis* culture-positive or culture-negative warthogs. Median and interquartile ranges are represented by the horizontal bars. The dotted lines represent the various published cut-off values used in other species (1, 2 and 4mm). ** indicates $p<0.01$, *** indicates $p<0.001$.

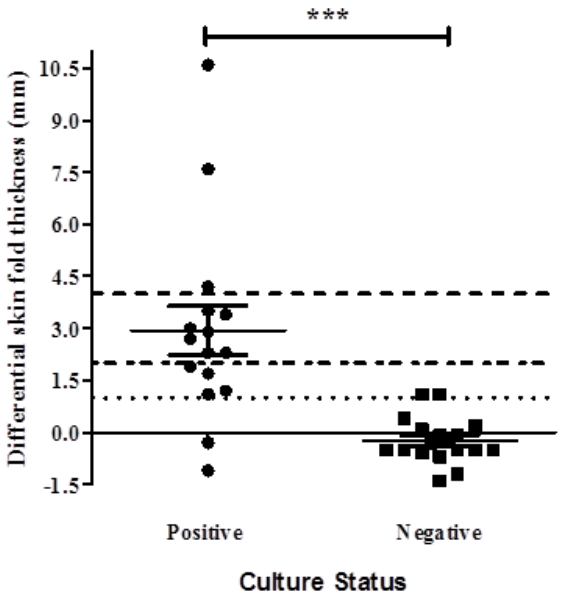


**Figure 5.3** Differential in skin fold thickness for *M. bovis*-infected and uninfected warthogs. Test value was calculated by subtracting the $PPD_a$ injection sites' skin fold thickness from that of the $PPD_b$ injection site after 72 h ($PPD_b$ - $PPD_a$). Median and interquartile ranges are represented by the horizontal bars. The dotted lines represent the various published cut-off values in other species (1, 2 and 4mm). *** indicates $p<0.001$.

**Table 5.3** Receiver operator characteristics curve analysis data for Δ $PPD_b$. Warthog specific cut-off values and respective sensitivity and specificity, with 95% CI in parentheses. Youden's index for each cut-off value is also indicated.

| Cut-off | Sensitivity | Specificity | Youden's index |
|---|---|---|---|
| > 0.1 | 100 (79-100%) | 56 (31-79%) | 49 |
| > 0.2 | 94 (70-100%) | 61 (36-83%) | 55 |
| > 0.3 | 94 (70-100%) | 67 (41-87%) | 60 |
| > 0.4 | 88 (62-98%) | 68 (41-87%) | 54 |
| > 0.5 | 88 (62-98%) | 72 (47-90%) | 60 |
| > 0.6 | 88 (62-98%) | 78 (52-94%) | 65 |
| > 0.7 | 81 (54-96%) | 79 (52-94%) | 59 |
| > 0.8 | 81 (54-96%) | 83 (59-96%) | 65 |
| > 1.0 | 75 (48-93%) | 84 (59-96%) | 58 |
| > 1.1 | 69 (41-89%) | 89 (65-99%) | 58 |
| > 1.3 | 69 (41-89%) | 94 (73-100%) | 63 |
| > 1.8 | 69 (41-89%) | 100 (81-100%) | 69 |
| > 2.4 | 63 (35-85%) | 100 (81-100%) | 63 |
| > 2.8 | 56 (30-80%) | 100 (81-100%) | 56 |
| > 3.0 | 50 (25-75%) | 100 (81-100%) | 50 |
| > 3.3 | 38 (15-65%) | 100 (81-100%) | 38 |
| > 3.8 | 25 (7-52%) | 100 (81-100%) | 25 |
| > 4.5 | 19 (4-46%) | 100 (81-100%) | 19 |
| > 5.9 | 13 (2-38%) | 100 (81-100%) | 13 |
| > 8.6 | 6 (0-30%) | 100 (81-100%) | 6 |

**Table 5.4** Receiver operator characteristics curve analysis data for $PPD_b - PPD_a$. Warthog specific cut-off values and respective sensitivity and specificity, with 95% CI in parentheses. Youden's index for each cut-off value is also indicated.

| Cut-off | Sensitivity | Specificity | Youden's index |
|---|---|---|---|
| > 0.2 | 88 (62-99%) | 78 (52-94%) | 65 |
| > 0.3 | 88 (62-99%) | 83 (59-96%) | 71 |
| > 0.8 | 88 (62-99%) | 89 (65-99%) | 76 |
| > 1.2 | 81 (54-96%) | 100 (81-100%) | 81 |
| > 1.5 | 75 (48-93%) | 100 (81-100%) | 75 |
| > 1.8 | 69 (41-89%) | 100 (81-100%) | 69 |
| > 2.1 | 63 (35-85%) | 100 (81-100%) | 63 |
| > 2.5 | 50 (25-75%) | 100 (81-100%) | 50 |
| > 2.8 | 44 (20-70%) | 100 (81-100%) | 44 |
| > 3.0 | 38 (15-65%) | 100 (81-100%) | 38 |
| > 3.2 | 31 (11-59%) | 100 (81-100%) | 31 |
| > 3.5 | 25 (7-52%) | 100 (81-100%) | 25 |
| > 3.9 | 19 (4-46%) | 100 (81-100%) | 19 |
| > 5.9 | 13 (2-38%) | 100 (81-100%) | 13 |
| > 9.1 | 6 (0-30%) | 100 (81-100%) | 6 |

## Discussion

This study shows that the ITT could distinguish between *M. bovis* culture-positive and negative warthogs from bTB endemic regions of South Africa, using both the SITT and CITT interpretations. Warthog specific cut-off values for the SITT and CITT were calculated to be ≥ 1.8 mm and ≥ 1.2 mm, respectively. The optimal ITT criterion for detection of infected warthogs in this study was the ≥ 1.2 mm cut-off for the CITT, which resulted in correct classification of 81% of culture-positive animals as CITT-positive and 100% of culture-negative warthogs as test negative. These results suggest that the interpretation of the ITT in this species should include the reaction to avian PPD (i.e., CITT) to identify the highest number of infected animals, while maintaining good specificity.

Importantly, diagnostic application in each species requires optimization and standardization of the ITT, as the injection site and dose may influence the delayed-type hypersensitivity response, with variable interpretation affecting test sensitivity (Palmer et al. 2001; Jaroso et al. 2010; Keet et al. 2010; Good et al. 2011). In this study, a double dose of tuberculin (0.2 ml PPD) was injected intradermally, caudal to each ear in warthogs, to minimise the chance of delayed-type hypersensitivity response failure due to dose, as is the case in lions and domestic cats (Keet et al. 2010).

The choice of ITT (SITT or CITT) is dependent on the prevalence and exposure of *M. bovis* as well as the presence of sensitising NTMs in a population (de la Rua-Domenech et al. 2006). The SITT is a simpler test, since it consists of one injection and measurement, although it lacks the discriminatory power of the CITT, where the response to $PPD_a$ identifies sensitisation to NTMs (de la Rua-Domenech et al. 2006). In this study, the response at the $PPD_a$ site was significantly less than that at the $PPD_b$ site, indicating that the increase in SFT at the $PPD_b$ site was a true measure of *M. bovis* infection and not a cross-reactive response to NTMs.

In many countries the initial recommendations that were in place for the ITT suggested the use of the single intradermal cervical test as the primary bTB screening test and the CITT as an ancillary test (de la Rua-Domenech et al. 2006). However, most of these countries have amended their regulations, as the CITT has been shown to be a more specific test than the SITT (Goodchild et al. 2015). Furthermore, our results showed that the CITT was more sensitive than the SITT (81% versus 69%, correctly identifying animals with a culture-positive result). This may be due to the more sensitive cut-off value of $\geq 1.2$ mm for the CITT compared to the $\geq 1.8$ mm cut-off for the SITT (Metz 1978; Greiner et al. 2000; de la Rua-

Domenech et al. 2006). It is important to note that neither the CITT nor SITT had any false positives in this study using these criteria.

Unfortunately, no biological test is perfect (i.e. has 100% sensitivity and specificity) and the CITT could not correctly classify all *M. bovis* culture-positive warthogs as test positive and at 81%, it is comparable to the sensitivity of the CITT in cattle (Hartnack and Torgerson 2012). The sub-optimal sensitivity could be due to various factors, for example, anergy, co-infection with NTMs, immunosuppression associated with nutritional, immobilization or transport stress, operator error, faulty equipment, or tuberculin not correctly administered intradermally (de la Rua-Domenech et al. 2006).

For disease surveillance, the cut-off value of a screening test should be set to optimize sensitivity and specificity, after considering the prevalence of disease and epidemiological factors as well as clinical and financial constraints (van Erkel and Pattynama 1998; Greiner et al. 2000). A cost-effective and logistically feasible method is required for disease surveillance in wildlife since access to laboratories may be limited. Although previous reports have shown that serological assays can be used to identify infected warthogs (Roos et al. 2016), these require laboratory equipment and techniques which may not be readily available. Therefore, the ITT may be an alternative screening test to laboratory-based assays in some situations.

One limitation of this study was that cut-off values and test specificity were determined using endemic controls rather than animals from a known *M. bovis* negative population. Therefore, future research should ideally include an unexposed population of warthogs to evaluate specificity and determine a diagnostic cut-off value for the ITT.

This study demonstrates that *M. bovis*-infected warthogs develop a specific delayed type hypersensitivity response to $PPD_b$, thus confirming the usefulness of the ITT for this species. Cut-off values determined by ROC curve analyses were able to distinguish between *M. bovis* culture-positive and culture-negative warthogs with good sensitivity and specificity. Interpretation of the ITT under the criteria followed for the CITT, allowed greater numbers of infected warthogs to be detected. Thus, the application of the ITT will be a valuable tool for disease surveillance in warthogs.

**Declarations**

Ethics approval and consent to participate:

This study received ethical clearance from the Stellenbosch University Animal Care and Use committee (SU-ACUD15-00029).

Consent for publication:

All authors accepted that this manuscript can be submitted.

Availability of data and material:

All data generated or analysed during this study are included in this published article Table 1.

Competing interests:

There is no conflict of interest to be reported by the authors.

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Authors' contributions:

ER, LM, SP and MM conceptualised and designed the project. ER, LM, MM, PB, GH were all part of sampling and sample processing. ER and FP were responsible for the data analysis and interpretation. SP, LM and MM supervised the findings and encouraged ER to investigate these. FP, PB, PH and RW supported the research with critical revision of the article. ER, SP, LM and MM drafted the article with all authors contributing substantially to the writing and revising of the manuscript. All authors approved the final version for submission (ER FP, PH, GH, RW, PH, SP, LM and MM).

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# Chapter 6

## Cytokine gene expression as a marker of antigen recognition in *Mycobacterium bovis*-infected warthogs (*Phacochoerus africanus*)

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**My contribution to this research article:**

Planning of project
Blood collection
Blood stimulation
Running all assays
Post mortem examinations
Tissue sample collection
Mycobacterial culturing
Speciation by PCR
Data interpretation
All statistical analyses
Writing of manuscript

**Abstract**

There is a lack of appropriate diagnostic tools in many wildlife species to detect *Mycobacterium bovis*-infected individuals, including warthogs (*Phacochoerus africanus*). Furthermore, few studies have investigated the use of cell-mediated immune (CMI) response assays in suids. Gene expression assays (GEA) utilising RNA, extracted from whole blood stimulated in the QuantiFERON®-TB Gold (In-Tube) (QFT) system, has shown great promise as a diagnostic tool across a wide range of species. In this study, the QFT system was used to stimulate and incubate 1ml of whole blood from *M. bovis*-infected and uninfected warthogs. RNA was extracted from stimulated blood and used to determine the relative gene expression of five reference (*B2M, H3F3A, LDHA, PPIA* and *YWHAZ*) and five target (*CXCL9, 10, 11, IFNG* and *TNFA*) genes through RT-qPCR. The most stably expressed reference gene was *H3F3A*, while all three *CXCL* genes were highly upregulated. *CXCL10* had the greatest level of upregulation with a mean fold increase of 4616 in *M. bovis*-infected warthogs. All the target genes were able to differentiate *M. bovis*-infected from uninfected warthogs. Importantly, the similarity between the warthog and pig sequences were greater than 97% for all reference and target genes, suggesting the potential use of the GEAs in other suids. We conclude that the GEA assay developed in this study could be useful for surveillance of *M. bovis*-infected warthogs.

**Highlights:**

- Developed an optimised gene expression assay in warthogs
- *CXCL* genes are highly upregulated compared to *IFNG* and *TNFA* in *M. bovis*-infected warthogs
- All target genes distinguished *M. bovis*-infected from uninfected warthogs

Keywords: Bovine tuberculosis, *CXCL10*, gene expression assay, *IFNG*, *Mycobacterium bovis*, suids, warthog

## Introduction

There are few antemortem diagnostic tests available to determine the *Mycobacterium bovis* infection status of *Phacochoerus africanus* (warthogs). The lack of appropriate tools is an obstacle to understanding the disease dynamics in warthogs which may serve as potential bovine TB (bTB) maintenance hosts in bTB-endemic areas (de Lisle et al. 2002; Roos et al. 2018a).

Serological assays have been used to distinguish between *M. bovis*-infected and uninfected warthogs (Roos et al. 2016). However, cell-mediated immune (CMI) responses have been shown to have greater sensitivity in many species (Maas et al. 2013). Most *in vitro* CMI assays are based on the stimulation of whole blood (WB) to detect antigen-specific production of cytokines, such as interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) (Waters et al. 2003; Cavalcanti et al. 2012). While these types of CMI assays have been used to detect *M. bovis*-infected individuals in species such as cattle and buffalo, very few have investigated their use in suids (de la Rua-Domenech et al. 2006).

Research has predominantly focused on measuring cytokine protein production for CMI response. However, recent studies have shown the utility of gene expression assays in various species for immune sensitization to *M. bovis* (Parsons et al. 2012; Olivier et al. 2015; Higgitt et al. 2017). These studies investigated the use of gene expression assays (GEAs) in wildlife, which included markers such as *IFNG* and those in the *CXCL* family, which are induced by

*IFNG* (Parsons et al. 2012; Olivier et al. 2015; Higgitt et al. 2017). Studies have also shown that *TNFA* is a potential diagnostic biomarker in *M. bovis*-infected cattle (Waters et al. 2003).

Mycobacterial antigens commonly used for *in vitro* stimulation of CMI responses to *M. bovis* are typically bovine and avian purified protein derivatives. However, specific peptides, such as those used in the QuantiFERON® TB Gold In-Tube (QFT) system also stimulate *M. bovis*-specific antigen responses in whole blood (WB) from a variety of animals (Parsons et al. 2011; Olivier et al. 2015; Higgitt et al. 2017; Roos et al. 2018b). The peptides in this system are early secretory antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10).

The aim of this study was to develop optimised GEAs for warthogs, targeting *IFNG* and *TNFA* as well as genes that are induced by *IFNG*, including *CXCL9, 10* and *11*. To achieve this, we determined the sequences of warthog gene transcripts for each reference and target gene, identified a suitable reference gene, and calculated the relative gene expression of our five target genes after immune activation to ESAT-6 and CFP-10 in whole blood from *M. bovis*-infected and uninfected warthogs.

## Materials and Methods

### *Animals, sampling and selection of study animals*

Heparinized WB and tissue samples were obtained opportunistically from 88 warthogs as previously described in detail (Roos et al. 2018b). Tissues were processed for mycobacterial culture (Goosen et al. 2014) and cultures were speciated using genetic region of difference analysis (Warren et al. 2006). Culture results were used to define animals as *M. bovis*-

infected or uninfected. The Stellenbosch University Animal Care and Use committee provided ethical approval for this study (SU-ACUD15-00029).

*Whole blood stimulation and RNA stabilization*

The QFT system (Qiagen, Venlo, Netherlands) was used to stimulate warthog WB. Briefly, 1 ml of WB was added to a Nil tube (QFT-Nil, containing saline), and a TB antigen tube (QFT-TB, containing ESAT-6/CFP-10 and TB7.7 peptides). As a positive control, pokeweed mitogen (PWM) (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 10 µg/ml was used instead of the QFT mitogen tube. The tubes were repeatedly inverted allowing contact of blood with their entire inner surface to ensure optimal antigen exposure. Following incubation for 24 hours at 37°C, blood was transferred to a 2 ml microcentrifuge tube and centrifuged at 800 × g for 10 min. The plasma was harvested, and the cell pellet was gently resuspended in 1.3 ml of RNAlater® (Ambion, Austin, TX, USA). Samples were then stored at -80°C until they were analysed.

*RNA extraction and cDNA preparation*

Five randomly selected RNAlater®-stabilised QFT-TB and QFT-Nil samples of *M. bovis*-infected and uninfected warthogs, as well as the PWM-stimulated samples of three randomly selected animals, were centrifuged at 15 000 × g for 2 min and the supernatant discarded. The remainder of the 1ml blood cell pellet was used to isolate RNA using the RiboPure™-Blood Kit (Ambion) according to the manufacture's guidelines. A single modification was made to the elution step; the volume of the elution solution was decreased to 60 µl. A NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to determine the RNA concentration of each sample. The QuantiTect Reverse Transcription (RT) kit (Qiagen), including a genomic DNA (gDNA) Wipeout step, was used to reverse transcribe

200 ng of RNA from each sample to cDNA in a final volume of 20 µl, as per manufacturer's guidelines.

*Sequencing of warthog mRNA transcripts*

To obtain warthog mRNA sequences of selected reference and target genes, degenerate primers were designed to anneal to the transcripts of interest (Table 6.1). The mRNA sequences of these transcripts for the pig and cow were obtained from the Ensembl Genome Browser (http://www.ensembl.org/index.html) and aligned using the Clustal Omega online tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). Using the online software Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi), primers were designed to anneal to sequences with greatest similarity within the untranslated regions. These were then used to amplify full mRNA transcripts from two randomly selected warthogs using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). For each transcript, 1 µl of cDNA was added to 12.5 µl of One*Taq*® Hot Start 2x Master Mix with Standard Buffer (New England BioLabs® Inc., Ipswich, MA, USA), 1 µl of each gene-specific forward and reverse primer (final concentration of 0.5µM; Integrated DNA Technologies, Coralville, IA, USA) and 9.5 µl nuclease-free water. The reaction was initiated at 94°C for 15 min, followed by 40 cycles of 94°C for 30s, a gene-specific annealing temperature for 30s (Table 1), and 68°C for 90s, and concluded with a final extension at 68°C for 5min. The PCR products were sequenced at the Central Analytical Facility (Stellenbosch University, South Africa) using a 3130xl Genetic Analyzer (Applied Biosystems), according to the manufacturer's guidelines and analysed using MEGA 7 (Kumar et al. 2016). The warthog mRNA sequences were submitted to the GenBank® genetic sequence database (http://www.ncbi.nlm.nih.gov/genbank/) (Table 6.1). The novel warthog sequences were

**Table 6.1** Warthog gene sequencing primers. Primers were designed from aligned pig and cow sequences of reference and target gene transcripts. This was done to obtain full warthog (*Phacochoerus africanus*) specific gene transcripts. Novel warthog gene transcripts were submitted to NCBI with accession numbers for each gene transcript included in the table. Primer pairs were designed to amplify the entire coding sequence and part of the 5′ and 3′ untranslated regions where possible.

| | Sequencing | | | |
|---|---|---|---|---|
| | Primer sequence (5'-3') | | | |
| Gene | Forward | Reverse | $T_{optimal}$°C | Accession numbers |
| *B2M* | ATTCCACCGCCAGCACCGCT | CCCCCTCTACATCTACCTGCT | 56 | Pending |
| *H3F3A* | ATGGCYCGWACMAAGCAGAC | CGRCGWGCYARCTGGATGTC | 56 | Pending |
| *LDHA* | GAAGTGCACTCCCGATTCCT | AGGCTGTCTTAACATTACTGCT | 56 | Pending |
| *PPIA* | TCGTGCTGCCTTGCA | GCTACAGAAGGAATGGTCTG | 51 | Pending |
| *YWHAZ* | RCASAACATCCAGTC | AARTGGTCTACTGTGTAAAT | 45 | Pending |
| *CXCL9* | ACAGRAGTGAYWYYRYYCTACCA | GCCMTCCYYTTYWGGAATTATTTCAG | 55 | Pending |
| *CXCL10* | CAKTSKGAGCCTRCMGCAGAAG | ARTCCAYGGACADTTAGGGCTTSA | 57 | Pending |
| *CXCL11* | TACTCCCTCCAAGAAGAGTATCA | AGCGTTCTTATTTCAGTATTCACAGT | 53 | Pending |
| *IFN-g* | TCTGGGCCTGATCGACTGTA | TTTGATCAATGAATCAATATTCCCCA | 52 | Pending |
| *TNF-a* | ACYTGARCCCYTCTGAAAA | AAACCAGAAGGRSRTGAG | 50 | Pending |

then compared to that of the pig and cow, to evaluate the similarity of the sequences between these species.

*qPCR optimization and design*

Using Primer3Plus and warthog-specific sequences, qPCR primers were designed to span putative exon-exon boundaries for each of the reference and target transcripts. All qPCRs were evaluated at annealing temperatures ranging from 58-62°C and primer concentrations ranging from 0.25-0.75 µM. The optimized reactions were done in triplicate using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) and consisted of 5 µl iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 0.5 µl of each gene-specific forward and reverse qPCR primer (at a final primer concentration of 0.5 µM; Integrated DNA Technologies), 1 µl of cDNA and 3 µl of nuclease-free water.

The reaction was initiated at 95°C for 30s, followed by 40 cycles of 95°C for 5s and either 60 or 62°C for 30s (Table 6.2), and concluded with a standard melt-curve analysis. To confirm the specificity of all qPCRs, products from two warthogs were visualized in 1% agarose gel by electrophoresis and sequenced as described above (Table 6.2). The melt-curve for each qPCR product was characterized and used to confirm qPCR specificity of subsequent reactions. All qPCRs had a no-template control, confirming the absence of non-specific amplification. In order to show the absence of amplifiable gDNA in RNA, qPCRs were performed with four randomly selected gDNA Wipeout-treated samples that had not been reverse transcribed. For each qPCR, quantification cycles ($C_q$) were automatically determined by the CFX Manager Software (Bio-Rad).

**Table 6.2** Warthog gene expression assay primers. Primers were designed to develop a gene expression assay, using full warthog (*Phacochoerus africanus*) specific gene transcripts. The qPCR spans putative exon-exon regions to increase the assay specificity. Sequences of qPCR products of two warthogs were aligned to the warthog specific gene transcripts to confirm the assay specificity. The homology to the pig sequence was also confirmed for each gene.

| | q-PCR | | | |
|---|---|---|---|---|
| | Primer sequence (5'-3') | | | |
| Gene | Forward | Reverse | $T_{optimal}$ | Pig homology |
| *B2M* | CTCACTGTCTGGCCTGGATG | GGCGGATGGAACCCAGATAC | 60°C | F: 100%; R: 100% |
| *H3F3A* | AAACAGATCTGCGCTTCCAG | ACGTTTGGCATGGATAGCAC | 60°C | F: 100%; R: 100% |
| *LDHA* | TGCAACATGGCAGCCTTTTC | ACAACCAGCCTAGAGTTTGC | 62°C | F: 100%; R: 100% |
| *PPIA* | TGAGTGGTTGGATGGCAAAC | TGGTCTTGCCATTCCTGGAC | 60°C | F: 100%; R: 100% |
| *YWHAZ* | TTCTGAACTCCCCAGAGAAAGC | GCGTGCTGTCTTTGTATGACTC | 62°C | F: 100%; R: 100% |
| *CXCL9* | TCATCTTCCTGACTCTGCTTGG | TGGATCATCCTTTGGCTGGTG | 62°C | F: 95%; R: 100% |
| *CXCL10* | CCCACATGTTGAGATCATTGCC | TCTCTCTGTGTTCGAGGAGATC | 60°C | F: 77%; R: 100% |
| *CXCL11* | AAAGCGGGAAGGTGTCTTTG | GGCATCTTCGTCCTTTATGTGC | 62°C | F: 100%; R: 100% |
| *IFN-g* | AGGCCATTCAAAGGAGCATG | AGTTCACTGATGGCTTTGCG | 60°C | F: 100%; R: 100% |
| *TNF-a* | GGCCCAAGGACTCAGATCATC | ATACCCACTCTGCCATTGGAG | 62°C | F: 100%; R: 100% |

*Data analysis*

The efficiencies of the qPCRs were determined by analysing a serial dilution (64 fold) of a pooled cDNA sample for each gene (Pfaffl 2001). To determine the intra-assay variability of each qPCR, the coefficient of variance was calculated for triplicate reactions. The stability of the selected reference genes were compared by analysing qPCR Cq values for QFT-Nil and QFT-TB samples of four randomly selected *M. bovis*-infected warthogs, using the geNorm applet in Microsoft Excel (Vandesompele et al. 2002) and the NormFinder Excel Add-In (Andersen et al. 2004). The most stable reference gene was used to further analyse the relative gene expression of the target genes.

The amplification efficiencies of the reference and the target gene need to be approximately equal to validate the use of the ΔΔCq method (Livak and Schmittgen 2001). The relative gene expression of each target gene was normalised by subtracting the Cq value of the most stable reference gene from the Cq value of the target gene. This was done to calculate the relative abundance of the mRNA for each sample (i.e. ΔCq). Thereafter, the ΔCq value derived from the QFT-Nil sample was subtracted from the ΔCq value derived from the QFT-TB sample for each animal (i.e. ΔΔCq). The relative fold change was used to derive an assay result in terms of the relative abundance of the target transcript ($2^{-\Delta\Delta Cq}$) (Livak and Schmittgen 2001). The $2^{-\Delta\Delta Cq}$ value was then log transformed for presentation purposes.

The data of the assay results for all target genes were then analysed in GraphPad Prism version 5 (GraphPad Software, March 2007). A Mann-Whitney *U* test was used to determine if there was a significant difference in the expression of each gene between *M. bovis*-infected and uninfected warthogs. Results with a p-value $< 0.05$ were considered statistically significant for this study.

**Results**

The median amount of RNA extracted from each sample was 2 700 ng (range: 366 - 11 772 ng) (Table 6.3). The 260/280 and 260/230 ratios had a median of 1.99 (range: 1.53-2.19) and 0.71 (range: 0.08-2.23) respectively (Table 6.3). All selected reference and target gene sequences derived from two warthogs showed greater homology with sequences of the domestic pig, as compared to those of the cow (Fig. 6.1, Table 6.1).

**Table 6.3** Summary of the RNA extracted from whole blood of warthogs after QFT-TB stimulation, QFT-Nil incubation and stimulation of PWM after a 24hr period, followed by RNAlater® stabilisation. RNA quality and quantity are represented as the 260/280 ratio which is the primary indicator of residual chemical contamination, the 260/230 ratio is a secondary measure of nucleic acid purity and the absolute RNA quantity extracted given as ng.

| Sample | 260/280 ratio | 260/230 ratio | RNA extracted (ng) |
|---|---|---|---|
| 15/248 Nil | 1,61 | 0,67 | 10 224 |
| 15/248 TB | 1,95 | 1,2 | 9 996 |
| 15/249 Nil | 1,99 | 2,14 | 11 772 |
| 15/249 TB | 2 | 1,59 | 7 434 |
| 15/262 Nil | 1,91 | 0,35 | 1 146 |
| 15/262 TB | 2,19 | 0,16 | 366 |
| 15/264 Nil | 1,55 | 0,14 | 894 |
| 15/264 TB | 1,53 | 0,08 | 480 |
| 15/270 Nil | 2,05 | 1,71 | 5 604 |
| 15/270 TB | 2,16 | 1,13 | 954 |
| 15/302 Nil | 2,05 | 0,52 | 1 326 |
| 15/302 TB | 1,94 | 0,46 | 2 694 |
| 15/308 Nil | 1,9 | 0,36 | 1 560 |
| 15/308 TB | 2,05 | 0,62 | 1 140 |
| 15/729 Nil | 1,94 | 0,64 | 7 032 |
| 15/729 TB | 1,99 | 0,71 | 4 326 |
| 15/W/021 Nil | 2,1 | 1,37 | 9 426 |
| 15/W/021 TB | 2,09 | 1,39 | 7 350 |
| 15/W/025 Nil | 2,12 | 1,55 | 10 998 |
| 15/W/025 TB | 2,1 | 2,23 | 5 016 |
| 15/140 PWM | 1,75 | 1,58 | 1 374 |
| 15/265 PWM | 1,75 | 0,61 | 708 |
| 15/266 PWM | 2,18 | 1,46 | 714 |

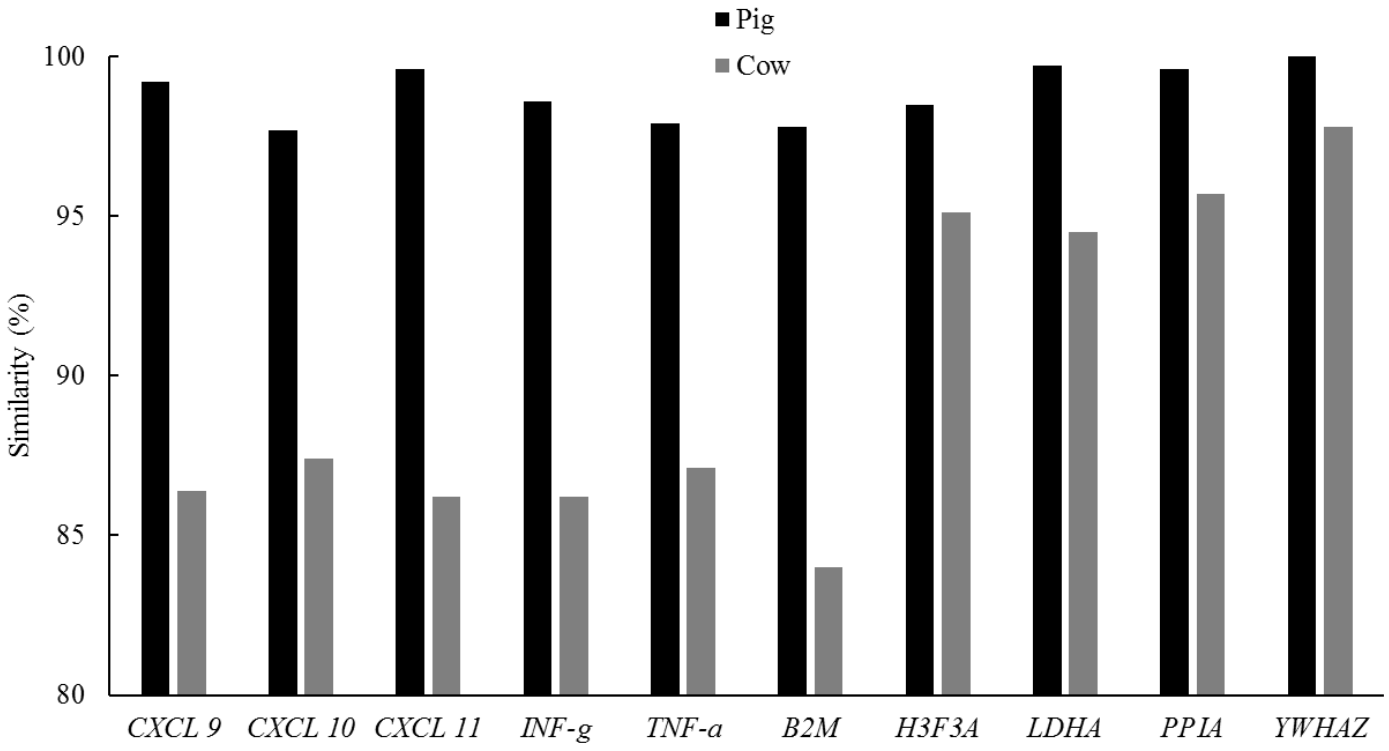


**Figure 6.1** A summary of the mRNA gene transcript similarity of the warthog with that of the domestic pig and domestic cow. The warthog transcripts were most like that of the domestic pig, with all transcripts having a similarity of more than 97%.

For all qPCRs, intra-assay variability was low (Table 6.4), amplification efficiencies ranged from 90 to 115%, and the efficiencies of all target gene qPCRs were not significantly different to that of the selected reference gene (data not shown). The qPCR products had characteristic melt curves and were confirmed to be specific by sequencing (data not shown). Furthermore, no products were formed in the no-RT control qPCRs as well as for all no template controls for each qPCR. Of the five reference genes, the *H3F3A* gene was the most stably expressed and chosen as the reference gene for further analyses (Fig 6.2).

For *M. bovis*-infected warthogs, all selected target genes showed significant up-regulation in response to antigen stimulation (Fig. 6.3). Of these, *CXCL9*, *CXCL10*, and *CXCL11* showed greatest up-regulation with mean fold increases of 371, 4616 and 260, respectively (Fig. 6.3).

The *M. bovis*-infected warthogs displayed significantly greater antigen-specific up-regulation of all target genes compared to uninfected animals (Fig. 6.3). Of the target genes, *CXCL10* showed the greatest differential response between *M. bovis*-infected and uninfected warthogs (Fig. 6.3).

**Table 6.4** Summary of qPCR parameters for each reference and target gene. Amplification efficiency of each gene transcript and the gene specific amplification factor are shown. All reference and target genes had a low intra-assay variability. The temperature that corresponded to each gene's specific derivative melt curve (DM) peak was used to confirm that the correct product was amplified in each qPCR.

| Reference gene | Efficiency (%) | Amplification factor | $R^2$ | Intra-assay variability (%) | DM peak (°C) |
|---|---|---|---|---|---|
| *B2M* | 103 | 2.03 | 0.99 | 0.5 | 82.5 |
| *H3F3A* | 105 | 2.05 | 0.99 | 0.4 | 80.5 |
| *PPIA* | 111 | 2.11 | 0.99 | 0.5 | 79.5 |
| *LDHA* | 114 | 2.14 | 0.99 | 0.7 | 77.5 |
| *YWHAZ* | 109 | 2.09 | 0.99 | 0.3 | 77.5 |

| Target gene | Efficiency (%) | Amplification factor | $R^2$ | Intra-assay variability (%) | DM peak (°C) |
|---|---|---|---|---|---|
| *CXCL9* | 97 | 1.97 | 0.99 | 0.8 | 77.0 |
| *CXCL10* | 108 | 2.08 | 0.99 | 0.5 | 76.5 |
| *CXCL11* | 105 | 2.05 | 0.99 | 0.7 | 78.0 |
| *IFN-g* | 115 | 2.15 | 0.98 | 1.0 | 78.5 |
| *TNF-a* | 101 | 2.01 | 0.98 | 1.0 | 81.5 |

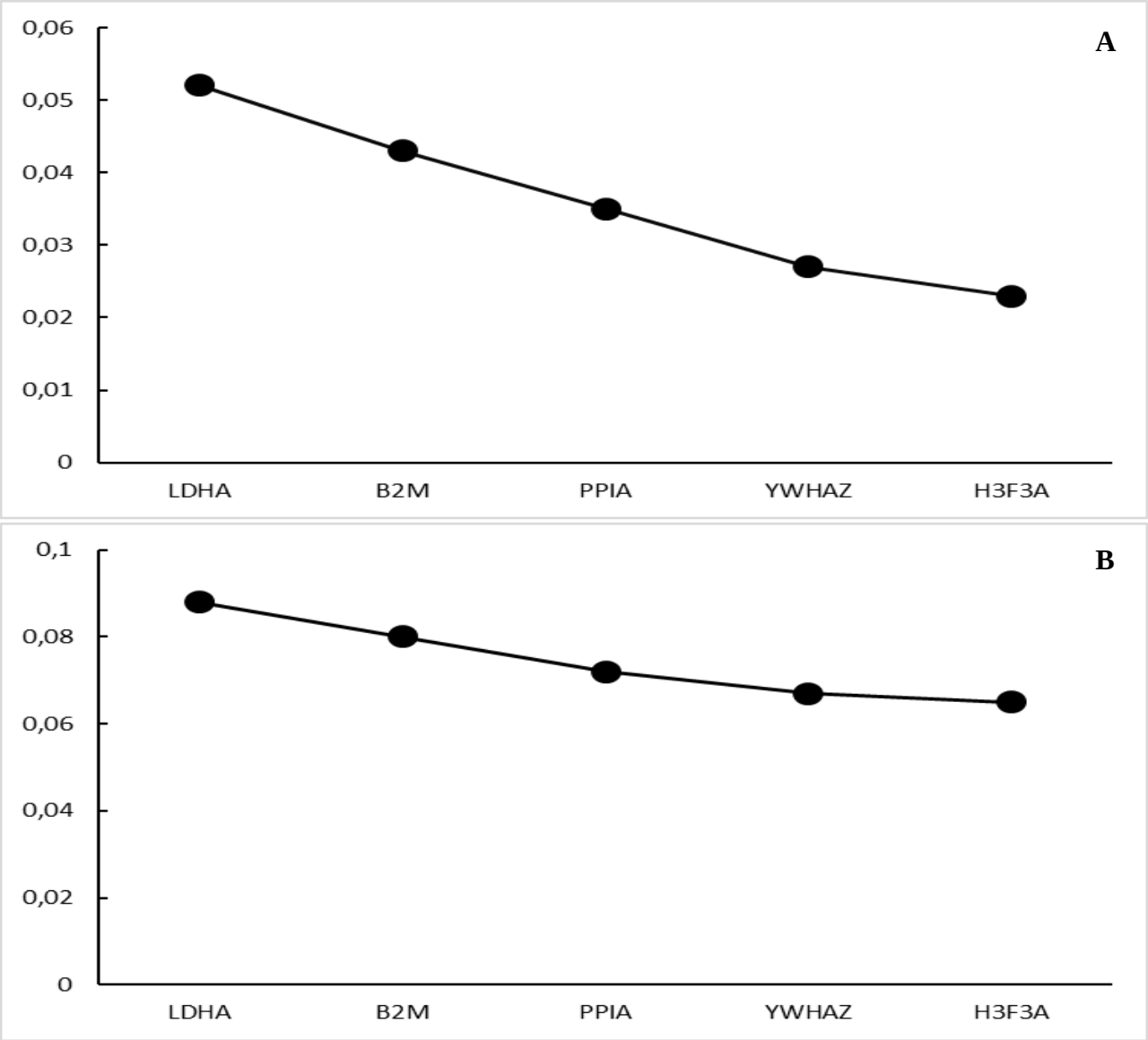


**Figure 6.2** Expression stability results from (A) NormFinder Excel Add-In and (B) geNorm Excel applet for five reference genes that were evaluated in warthogs. Stability was determined using QFT-TB stimulated and QFT-Nil incubated samples from four randomly selected warthogs.

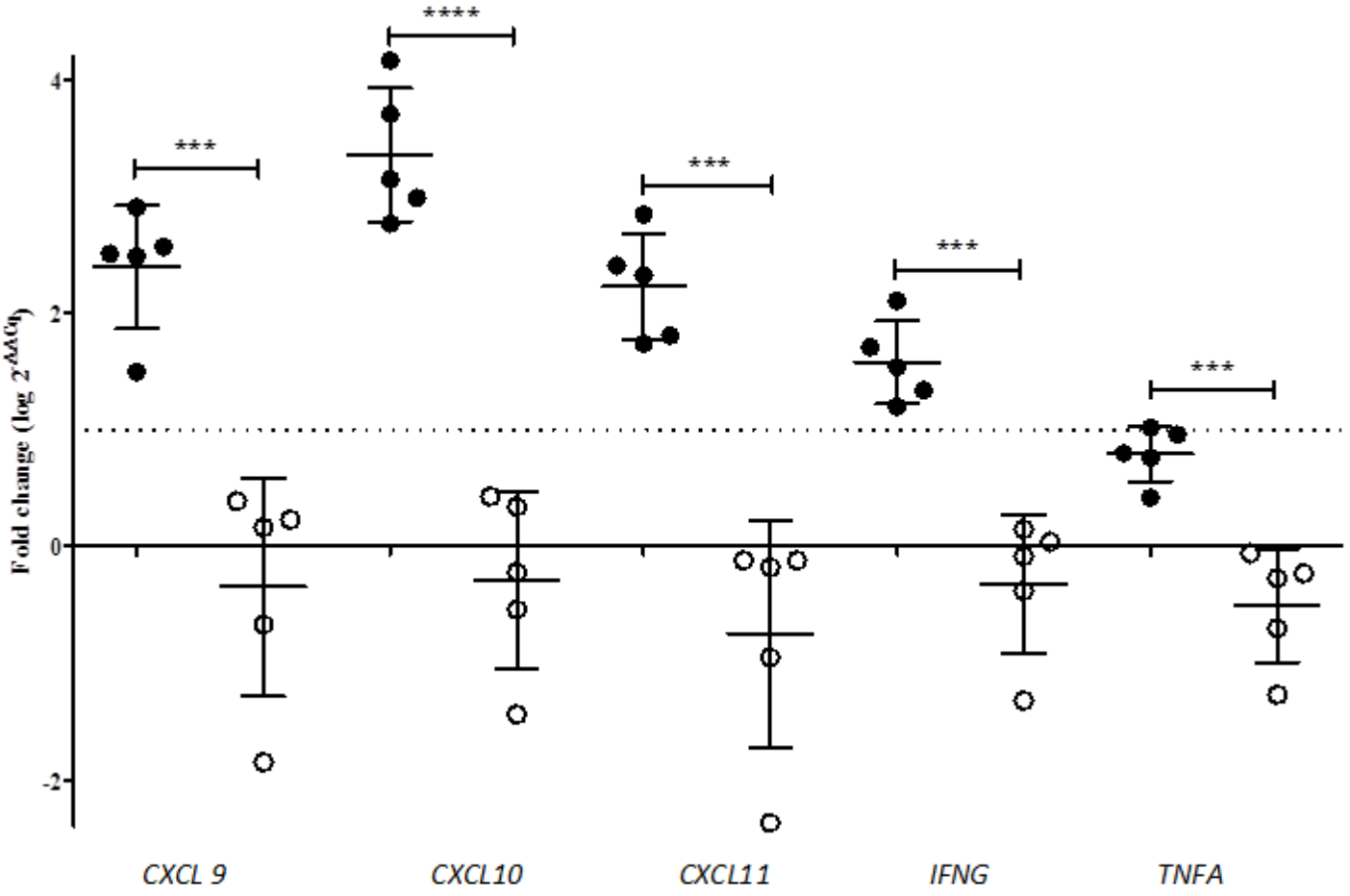


**Figure 6.3** Change in gene expression of five target genes for *M. bovis*-infected (●) and uninfected (○) warthogs. All targets were normalised against the most stable reference gene, H3F3A. The error bars represent the mean and standard deviation with the dotted line representing a fold change of 10. *** indicates $p < 0.001$, **** indicates $p < 0.0001$.

## Discussion

In this study, novel cytokine gene expression assays were developed that could distinguish between *M. bovis*-infected and uninfected warthogs. Among the five reference genes evaluated, *H3F3A* was the most stably expressed. Although all target genes (*CXCL9,10,11, IFNG, TNFA*) were able to distinguish *M. bovis*-infected from uninfected warthogs, *CXCL10* had the greatest antigen-specific up-regulation.

While both *IFNG* and *TNFA* were significantly upregulated in *M. bovis*-infected compared to uninfected warthogs, their level of up-regulation was much lower than that of the *CXCL* genes. This might be due to the incubation time of the QFT-tubes, which was set at 24 h for logistical reasons. In humans, peak mRNA levels for *IFNG* are detected after 4-6 h of incubation after which it returns to nearly background levels after 24 h (Kawabuchi et al. 2004; Kim et al. 2013). Nonetheless, other studies have shown that measurement of *IFNG* expression at 20 h can still distinguish *M. bovis*-infected from uninfected animals (Parsons et al. 2012; Olivier et al. 2015). Studies in lions and hyena, in which WB was incubated for 20 h in QFT tubes, showed similar results to our study where *IFNG* was expressed at a much lower level than the *CXCL* genes (Olivier et al. 2015; Higgitt et al. 2017). Therefore, investigating cytokine gene transcripts that are induced by *IFNG* could identify more suitable diagnostic candidates, since it would be expected that these biomarkers would be induced and increase during the longer incubation period (Higgitt et al. 2017). The abundance of IFN-γ induced markers is higher since they are produced by a greater number of cell types such as lymphocytes and monocytes in antigen stimulated WB (Ruhwald et al. 2007, 2008).

Studies have shown that *CXCL9* can be used in lions as a diagnostic biomarker and *CXCL11* is significantly up-regulated in *M. bovis* exposed hyenas (Olivier et al. 2015; Higgitt et al. 2017). In this study, the greatest differential expression between *M. bovis*-infected and uninfected warthogs was observed with *CXCL10*, which encodes the cytokine IP-10. These results suggest this chemokine gene may be a potential biomarker for *M. bovis* infection in warthogs and is supported by a previous study showing the utility of antigen-specific stimulation of IP-10 in this species (Roos et al. 2018b).

The mRNA gene transcript homology between the warthog and the domestic pig also indicates that the *CXCL* GEA could be employed for detection of antigen-specific CMI responses in other suids, such as domestic pigs and wild boar. All the q-PCR primers span putative exon-exon boundaries and match the mRNA sequence of *Sus scrofa*. Although not part of this study, the next step to using the *CXCL* GEA as a diagnostic test would be to establish appropriate diagnostic cut-off values for these genes in suids.

A limitation of GEA studies is their dependence on a good quality RNA yield. Even though there was some variation in RNA yield between individual warthogs, this could have been due to differences in sample processing or individual animal variability (Whitney et al. 2003; Chomczynski et al. 2016). Although our study had lower yields of total RNA compared to human studies (6 690 to 22 720 ng/ml of blood) (Chomczynski et al. 2016), the yield was comparable to that found in other wildlife species such as buffalo, lion and hyena (240 to 6 000 ng/ml of blood) (Parsons et al. 2012; Olivier et al. 2015; Higgitt et al. 2017). A further limitation in this study was that the optimum incubation time for warthog WB was not determined prior to measuring the gene expression of each target gene (Kasprowicz et al. 2011; Kim et al. 2013).

Future studies should focus on determining cut-off values and the diagnostic performance of each cytokine. The optimum antigen incubation time of WB should also be determined for this species.

In conclusion all five target genes (*CXCL9, 10, 11, IFNG and TNFA*) were significantly upregulated in blood in response to antigen stimulation in *M. bovis*-infected warthogs, with *CXCL10* showing the greatest upregulation. The high homology between warthog and pig

sequences suggest that these reference and target genes could potentially be used in GEAs for other suids. Thus, GEAs targeting the *CXCL* genes need to be further investigated as potential diagnostic biomarkers for *M. bovis* infection in suids.

## Conflict of interest

There is no conflict of interest to be declared by the authors regarding this publication.

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# Chapter 7

## Whole genome sequence analysis of *Mycobacterium bovis* isolated from naturally infected warthogs (P*hacochoerus africanus*)

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**My contribution to this research article:**

Planning of project
Post mortem examinations
Tissue sample collection
Mycobacterial culturing
Speciation by PCR
Data interpretation
Statistical analyses
Writing of manuscript

**Abstract**

*Mycobacterium bovis*, a pathogenic mycobacteria, has an extensive host range that includes livestock and wildlife. It shares a high genetic similarity with *M. tuberculosis* as it has >99.95% homology between nucleotide sequences. Various genotyping methods (i.e. spoligotyping, variable number tandem repeat typing (VNTR) and RD-analysis) have been used to describe the molecular epidemiology of *M. bovis*. However, advances in whole genome sequencing (WGS) have increased the resolution and enabled the detection of a broad range of genomic variants. Therefore, WGS can resolve the unique problem faced by traditional genotyping methods that struggle to differentiate between *M. bovis* strains, since they lack substantial genetic diversity. This study investigated the spoligotype diversity of 19 *M. bovis* isolates of warthogs from Kruger National Park, Marloth Park (MP) and uMhkuze Nature Reserve (MZ) and WGS analysis was performed on 8 of these isolates (MP = 7 and MZ = 1). A further 106, previously published, *M. bovis* isolates were included in the WGS analysis. Of these 12 isolates were from various other species in KNP, which shares a border with MP. WGS analysis was able to determine that the KNP and MP isolates form two distinct clades, although they had the same spoligotype and *in silico* RD-analysis (RDbovis(c)_Kruger). In addition, WGS was able to confirm the *M. bovis* diversity seen in MP. Therefore, the higher resolution from WGS helped to increase the insight into the molecular epidemiology of the seven *M. bovis* isolates from MP and their relation to isolates from KNP. The study also highlighted the care that should be taken when describing transmission events when the genotype is only based on spoligotyping, VNTR and RD-analysis.

**Highlights:**

- High diversity of *M. bovis* strains identified by spoligotyping in an isolated warthog population
- WGS confirms diversity of *M. bovis* in an isolated population of warthogs
- WGS identified two distinct clades of *M. bovis* isolates compared to a single strain identified by spoligotyping

Keywords: genetic diversity, *Mycobacterium bovis*, spoligotyping, whole genome sequencing

## Introduction

*Mycobacterium bovis* is part of the *Mycobacterium tuberculosis* complex (MTBC), a group of pathogenic mycobacteria that infect a wide range of hosts (Smith et al. 2006; Maas et al. 2013). The MTBC has a high genetic similarity, with *M. bovis* sharing >99.95% similarity with *M. tuberculosis* at the nucleotide level (Garnier et al. 2003). Bovine tuberculosis (bTB) is caused by infection with *M. bovis* in animals, as well as zoonotic tuberculosis in humans who are exposed to infected animals or products, such as consuming raw unpasteurised milk of cows, goats, and camels (Hlavsa et al. 2008; Ereqat et al. 2013; Grandjean-Lapierre et al. 2018).

To understand the molecular epidemiology of *M. bovis*, various tools have been used to describe the relatedness of strains. Two commonly used genotyping methods are spoligotyping and variable number tandem repeat (VNTR) typing (Smith et al. 2006). Whole genome sequencing (WGS) as a genotyping tool has increased resolution compared to these traditional strain typing methods, and has recently been employed to give novel insight into the molecular epidemiology of *M. bovis* (Joshi et al. 2012; Dippenaar et al. 2017; Crispell et

al. 2017). This technique provides the means to differentiate isolates on a nucleotide level which allows detection of a broad range of genomic variants, including single nucleotide polymorphisms (SNP), insertions or deletions (Rausch et al. 2012; Walker et al. 2013; Dippenaar et al. 2015).

Studies have shown that the diversity of *M. bovis* strains, in naturally infected systems, is often geographically localized, which is indicative of genetic fixation and leads to a single strain becoming dominant (Smith et al. 2006; Dippenaar et al. 2017; Crispell et al. 2017). The potential lack of genetic diversity poses a problem for traditional genotyping methods, which do not have as high resolution as WGS, resulting in identification of these strains as a single spoligotype or VNTR pattern (Smith et al. 2006; Crispell et al. 2017). This characterization may be misleading in terms of determining transmission and evolution within the system.

The aim of this study was to describe the diversity of *M. bovis* isolated from naturally infected warthogs. Moreover, the study compared the use of spoligotyping and WGS to describe the molecular epidemiology of *M. bovis* in this species in endemic areas of South Africa.

## Materials and Methods

### *Sample collection and mycobacterial cultures*

Warthog tissue samples were collected from 67 warthogs during routine disease surveillance in Kruger National Park (KNP) from Skukuza (n = 5) and Satara (n = 19) camps; Marloth Park (MP; n = 24) in Greater KNP (Fig. 7.1), and uMhkuze Nature Reserve (MZ; n = 21), which is more than 200km to the south of MP. Lymph nodes were sampled from all animals and frozen at −20°C until processed (Roos et al. 2016). Mycobacterial cultures were

performed in a BACTEC™ MGIT™ 960 system (BD Biosciences, New Jersey, USA) as described by Goosen et al. (2014). The Stellenbosch University Animal Care and Use committee approved this study (SU-ACUD15-00029).

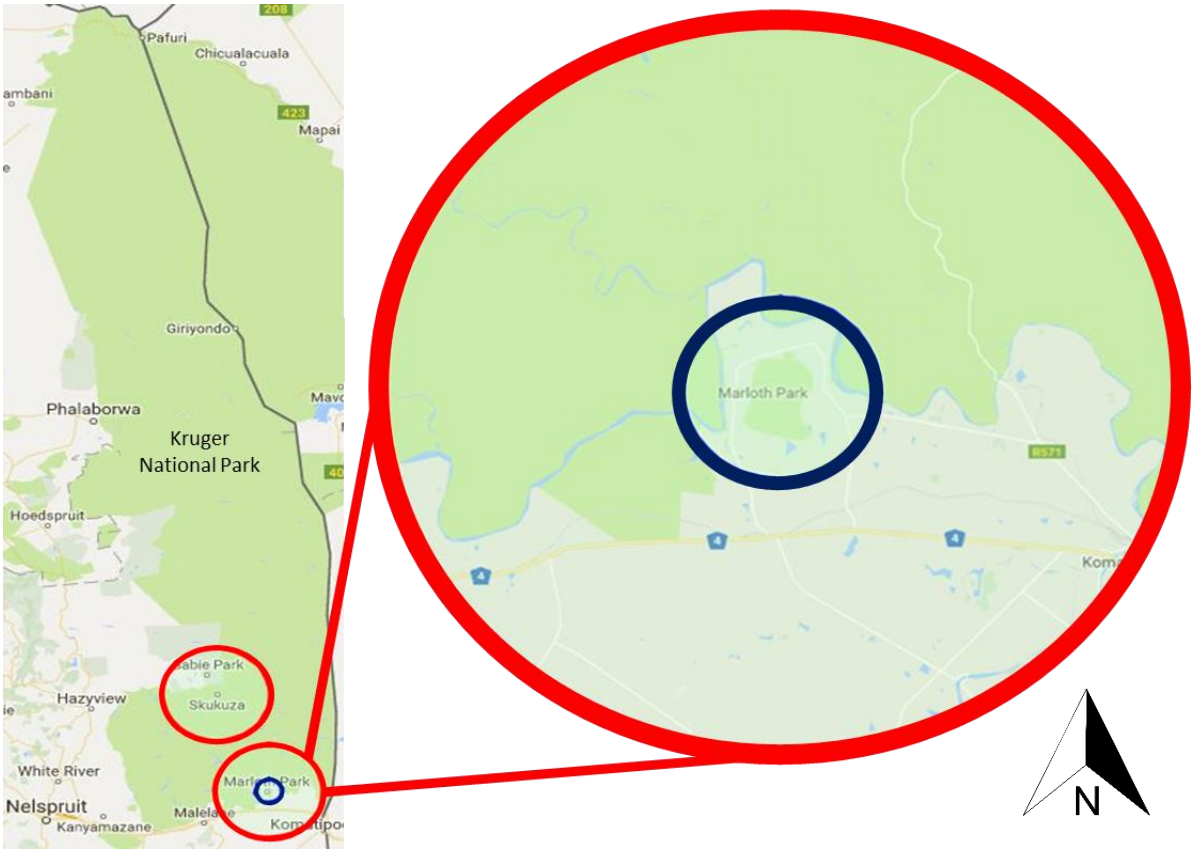


**Figure 7.1** Map of Kruger National Park (KNP) and the surrounding areas. The top red circle represents a 20km radius from the centre of Skukuza rest camp in KNP. While the bottom red circle represents a 20km radius from the centre of Marloth Park (MP). The blue circle represents a 5km radius from the centre of MP. A geographical barrier, the Crocodile River, is seen on the enlarged image. The map also shows that MP borders KNP and the Crocodile River to its western and northern to eastern border.

*Speciation and culture of M. bovis isolates*

Samples with positive growth in MGIT were genetically speciated using 16S DNA sequencing and the regions of difference (RD) analysis (Leclerc et al. 2000; Warren et al. 2006a). Isolates identified as *M. bovis* were inoculated onto Middlebrook 7H11 medium (BD

Biosciences) agar plates supplemented with 0.5% sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), as per manufacturer's instructions and incubated for 6-8 weeks. Thereafter, isolated colonies were harvested from plates and subjected to DNA extraction (Warren et al. 2006b). Bacterial isolates were subjected to spoligotyping twice, once as a crude boiled culture from the MGIT, after *M. bovis* RD confirmation, and once using DNA extracted from colonies on plates (Kamerbeek et al. 1997). All spoligotypes from this study were compared to the database on Mbovis.org (https://www.mbovis.org/database. php).

*Whole genome sequencing*

Extracted DNA from eight *M. bovis*-isolates (Table 7.1) were subjected to whole genome sequencing (USDA, Animal and Plant Health Inspection Service, National Veterinary Services Laboratories, Ames, IA, USA) on the Illumina MiSeq platform (Illumina, San Diego, California, USA) using a paired end approach (2 × 250bp) with the 500-cycle MiSeq Reagent Kit v2 (Illumina). Library preparation was done by adding 1µg DNA to the Nextera XT DNA Library Prep Kit (Illumina), following the manufacturer's instructions. Sequences were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/) (Table 7.1), project accession: PRJEB27859.

**Table 7.1** Sequences of *M. bovis* isolates from warthogs in Marloth Park (MP) and uMhkuze (MZ) included in the analyses.

| Isolate name | Genbank accession number |
|---|---|
| *M. bovis* MP1 | Pending |
| *M. bovis* MP4 | Pending |
| *M. bovis* MP7 | Pending |
| *M. bovis* MP9 | Pending |
| *M. bovis* MP10 | Pending |
| *M. bovis* MP11 | Pending |
| *M. bovis* MP20 | Pending |
| *M. bovis* MZ17 | Pending |

*WGS data and phylogenetic analysis*

*M. bovis* sequences from this study and previously published WGS data for *M. bovis* isolates (n=106) from South Africa (Dippenaar et al. 2017) were analysed using various open source software (Black et al. 2015; Dippenaar et al. 2015). Briefly, the quality of the sequence data was assessed using FASTQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc), next reads were trimmed with Trimmomatic based on their quality and from the ‘3 end (Bolger et al. 2014). Sequence reads were then aligned to the reference genome of *M. tuberculosis* H37Rv (GenBank NC000962.3) to allow the detection of genomic variants. Novoalign (Novocraft, Selangor, Malaysia), Burrows-Wheeler Aligner (BWA) (Li and Durbin 2009), and SMALT (Ponsting and Ning 2010) were used as the alignment algorithms. The average depth of coverage for our sequenced *M. bovis* isolates had to be more than >75× for analysis. The Genome Analysis Tool Kit (GATK) was used with all the alignment files of the different mapping algorithms (McKenna et al. 2010) to identify single nucleotide variants, small insertions and deletions (Indels). Only variants that were present in all three alignments, according to GATK, overlapping in their position and base identity, were used and variants in *pe*/*ppe* family genes, repeat regions, insertion sequences and phages were excluded (Coscolla et al. 2013). *In silico* spoligotyping was performed by using Spotyping (Xia et al. 2016). Furthermore, *in silico* RD-analysis was performed by viewing all the *M. bovis* isolates against the reference H37Rv *M. tuberculosis* genome in Artemis (Carver et al. 2005), by identifying the presence or absence of previously published RDs (Dippenaar et al. 2017).

High confidence variable sites, including coding and non-coding SNPs were concatenated to generate a multi-FASTA file. The optimal substitution model was determined with Modelgenerator (Felsenstein 1989; Keane et al. 2006). From this analysis, the general time reversal (GTR) model was chosen as it describes the substitution pattern that best fit this

dataset, and since it had the lowest model score according to the Bayesian information criterion's (BIC) hierarchical likelihood ratio test. Following the selection of the best fit model, a maximum likelihood (ML) phylogeny was constructed using the Randomized Axelerated Maximum Likelihood (RaxML) with 1000 bootstrap pseudo-replicates (Stamatakis 2006, 2014). Gaps and missing data were not included in the analysis.

The SNPs of isolates from this study were compared with one another to determine their pairwise distance. Isolates from various host species within KNP were also selected and compared to isolates from MP to determine the number of variants between these isolates.

**Results**

*Spoligotyping and* in silico *RD-analysis*

Five different spoligotype patterns were observed from the 19 warthog *M. bovis* isolates that were analysed (Table 7.2). The spoligotype pattern for the crude boiled MGIT culture and extracted DNA samples were identical matches for all eight isolates that had WGS data (data not shown). Although the seven WGS *M. bovis*-isolates from MP were identified as SB0121 by *in silico* spoligotyping, standard spoligotyping of the crude boiled MGIT cultures and the extracted DNA samples identified isolates MP9, MP11 and MP20 as SB1275. The one *M. bovis* isolate from MZ was identified as SB0140 by *in silico* spoligotyping, which matched the spoligotype from culture and DNA samples.

All *M. bovis* isolates from MP had the RDbovis(c)_Kruger deleted and RD17 present compared to the *M. bovis* isolate from MZ which has the RDbovis(c)_Kruger present and RD17 deleted (Table 7.3). The *M. bovis* isolates from MP had the same deletion profile as isolates from KNP (Table 7.3) (Dippenaar et al. 2017).

**Table 7.2** Spoligotype patterns for 19 warthog *M. bovis* isolates. Samples were analysed as crude boiled MGIT culture and extracted DNA, with both samples having the same spoligotype from individual warthogs.

| Pattern number | N | Spoligotype pattern |
|---|---|---|
| SB0120 | 1 |  |
| SB0121 | 8 |  |
| SB0140 | 1 |  |
| SB1275 | 8 |  |
| SB1388 | 1 |  |

*Phylogenetic analysis*

All eight *M. bovis* isolates that were sequenced had an average depth of coverage >75×, with >98.5% of reads mapping to the H37Rv reference genome. A maximum likelihood phylogenetic relationship was constructed, using concatenated sequences with 36,841 high-confidence variable sites which were from coding and non-coding SNPs (Fig. 7.2). These concatenated sequences were from isolates sequenced for this study (Table 7.1) and those previously published (Dippenaar et al. 2017).

The phylogenetic analysis confirmed that isolates from MP and MZ were within the *M. bovis* cluster of the MTBC. SNP distances between 6 warthog isolates from MP were between 3 and 23 variants (Fig. 7.3 and Table 7.4). The isolate from warthog MP7 had a variant distance of >49 variants compared to the rest of the MP isolates (Fig. 7.3 and Table 7.4). The variant distances between MP and KNP isolates were 34-57 variants (Fig. 7.3 and Table 7.4). The MP and KNP clades differed by 5 unique variants (Fig. 7.3). The MZ17 sample had a variant distance of 425-449 compared to isolates from the MP and KNP clades (Fig. 7.2 and Table 7.4).

**Table 7.3** Deleted regions of *M. bovis* isolates from Kruger National Park (KNP), Marloth Park (MP) and uMhkuze Nature Reserve (MZ). The start and end positions are compared to the reference genome of H37Rv *M. tuberculosis*. The length of each deletion and the open reading frame affected are indicated for each isolate.

| Deleted region | Start | End | Length (bp) | Affected ORF(s) | KNP1081 | KNP440 | KNP659 | KNP747 | MP1 | MP4 | MP7 | MP9 | MP10 | MP11 | MP20 | MZ17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RD10 (Brosch et al., 2002) | 264500 | 266500 | 1.9 kb | Rv0221-Rv0223 | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| RD13 Brosch et al., 2002) | 1402778 | 1406084 | 3 kb | Rv1255c-Rv1257c | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| RDbovis(c)_Kruger | 1523189 | 1547066 | 23,877 | Rv1355c-Rv1374c | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | no |
| RD4 (brosch et al., 2002) | 1696016 | 1708749 | 12733 | Rv1505c-Rv1516c | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| RDbovis(c)_wbbl2 | 1720074 | 1720719 | 645 | Rv1525 | no | no | no | no | no | no | no | no | no | no | no | no |
| RD17 | 1768073 | 1768880 | 807 | Rv1563c | no | no | no | no | no | no | no | no | no | no | no | yes |
| RD3 (Brosch et al., 2002) | 1779500 | 1788500 | 9.2 kb | Rv1573-Rv1586 | no | no | no | no | no | no | no | no | no | no | no | no |
| RD7 Brosch et al., 2002) | 2207500 | 2220500 | 12.7 kb | Rv1964-Rv1977 | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| RD9 (Brosch et al., 2002) | 2330000 | 2332000 | 2 kb | Rv2072-Rv2075 | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| RD5 (Brosch et al., 2002) | 2625888 | 2636963 | 9 kb | Rv2346c-Rv2353c | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| RD11 (Brosch et al., 2002) | 2970016 | 2980971 | 10955 | Rv2645-Rv2659c | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| RDbovis(c)_virS | 3447448 | 3451242 | 3,794 | Rv3082c-Rv3085 | no | no | no | no | no | no | no | no | no | no | no | no |
| RD12 (Brosch et al. 2002) | 3483974 | 3487711 | 2.8 kb | SseC-Rv3121 | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| RDbovis(d)_1160_2 | 3823567 | 3825361 | 1,794 | Rv3403c-Rv3406 | no | no | no | no | no | no | no | no | no | no | no | no |
| RD6 (Brosch et al., 2002) | 3842845 | 3847543 | 4698 | Rv3425-Rv3428c | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| N-RD17 | 3897069 | 3897783 | 715 | Rv3479 | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| RD8 (Brosch et al., 2002) | 4056800 | 4062700 | 5.9 kb | Rv3617-Rv3618 | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| RDbovis(d)_buff2 | 4142144 | 4143784 | 1,640 | Rv3699-Rv3700c | no | no | no | no | no | no | no | no | no | no | no | no |
| N-RD25 | 4189605 | 4190757 | 1153 | Rv3738c-Rv3739c | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| RD1 (Brosch et al., 2002) | 4348811 | 4359797 | 9.5 kb | Rv3871-Rv3879c | no | no | no | no | no | no | no | no | no | no | no | no |
| RDbovis(a)_Δpan | 4370865 | 4375133 | 4,268 | Rv3887c-Rv3892c | no | no | no | no | no | no | no | no | no | no | no | no |
| Rdpan | 4371139 | 4373353 | 2214 | Rv3887c-Rv3889c | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| Novel | 4376917 | 4377673 | 756 | Rv3894c | no | no | no | no | no | no | no | no | no | no | no | no |

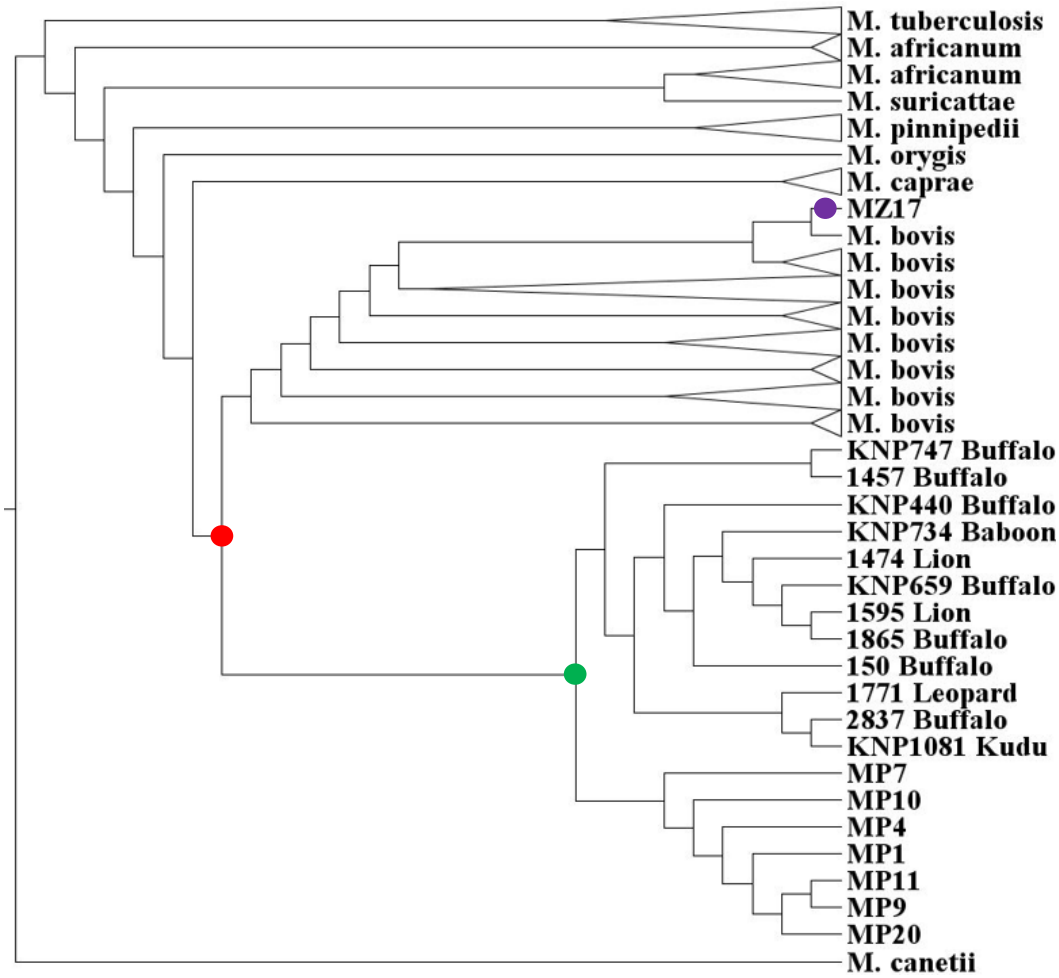


**Figure 7.2** The maximum likelihood method was used for the molecular phylogenetic analysis. A bootstrap consensus tree was inferred from 1000 replicates of the 36,841 variable positions between the concatenated sequences. The analysis represents the evolutionary history of the taxa (Felsenstein 1989). The phylogenetic tree was produced by RaxML, based on the variable positions that were identified with respect to the M. tuberculosis H37Rv reference sequence (Stamatakis 2006, 2014). The *M. bovis* clade is indicated with a red dot (●) and the split between the Kruger National Park (KNP) and Marloth Park (MP) strains, with the common ancestry indicated by green dot (●) on the phylogenetic tree. The sequence from uMhkuze Nature Reserve (MZ) is also part of the *M. bovis* clade (●).

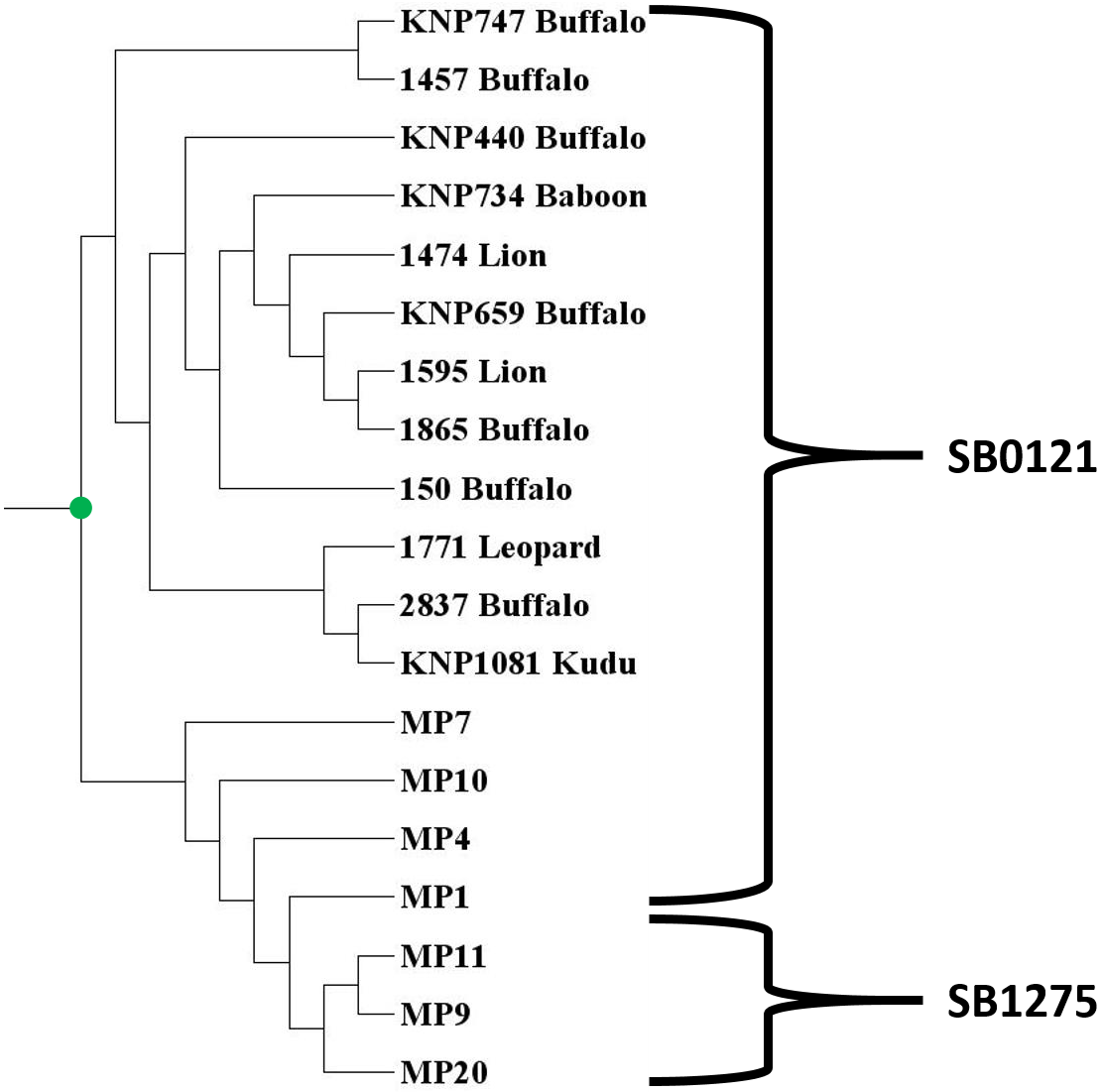


**Figure 7.3** Enlarged view focusing on the relationship between Kruger National Park (KNP) and Marloth Park (MP) isolates of the phylogenetic tree that was produced by RaxML in Figure 7.2. The *M. bovis* split between the KNP and MP strains with the common ancestry indicated by green dot (●) on the phylogenetic tree. Spoligotyping results indicated on the right-hand side.

**Discussion**

The aim of the study was to describe the genetic diversity of *M. bovis* isolates that were collected from naturally infected warthogs. The results showed that *M. bovis* isolates from MP, analysed by WGS, are distinctly different from isolates from KNP, although they do

share a common ancestor. These isolates were from close geographical locations, with KNP and MP only separated by a river. Therefore, the isolates from these warthogs were expected to be the same. The study demonstrated that the increased resolution of WGS could be used to describe the genetic diversity of *M. bovis*, even among those within the same spoligotype. Moreover, the diversity between isolates from MP, detected by spoligotyping, was confirmed by WGS. In addition, and most importantly, WGS identified two distinct clades of *M. bovis* in the GKNP, a "KNP-strain" and "MP-strain" which, by traditional genotyping methods would have been missed (Fig. 7.3).

Previous work that described isolates from the GKNP area used a combination of spoligotyping and VNTR-typing (Michel et al. 2009; Musoke et al. 2015; Hlokwe et al. 2016). With continually decreasing cost and increasing availability of WGS, the use of this technique, with higher resolution and greater discriminatory power, will improve identification of distinct genetic profiles of *M. bovis* isolates (Biek et al. 2012; Walker et al. 2013; Crispell et al. 2017). Furthermore, the single nucleotide resolution of WGS will facilitate improved determination of case links based on strain divergence (Rausch et al. 2012; Walker et al. 2013). In this study, screening for distinct genetic profiles using RD-analysis (RDbovis(c)_Kruger) would not have differentiated the MP and KNP isolates (Caimi et al. 2001; Warren et al. 2002; Dippenaar et al. 2017).

Warthog *M. bovis* isolates from MP were distinct from KNP isolates, based on WGS analysis, although some of the MP isolates had the same spoligotype pattern, SB0121, as the KNP isolates. When spoligotypes of MP isolates were determined using crude boiled cultures, the dominant strains emerged as SB0121 and SB1275, with eight isolations of each strain. The contradictory result for the *in silico* spoligotyping (all determined to be SB0121)

could be due to the strict filtering that was applied during the alignment and genome analysis, thus, potentially filtering out some of the distinguishing markers. In addition, strains SB0120 and SB1388 were also found in MP, suggesting a high diversity of *M. bovis* in this population. The single isolate from MZ belonged to the dominant strain SB0140, circulating in Kwa-Zulu Natal (where MZ is located).

Although identical strains may suggest transmission between two hosts (Musoke et al. 2015; Hlokwe et al. 2016). Care should be taken in defining "identical strains" based on VNTR and/or spoligotyping when considering transmission events. The higher resolution of WGS has shown that it can resolve transmission events of *M. bovis*, while other genotyping methods were unable to differentiate closely related isolates (Crispell et al. 2017). Unfortunately, directionality of transmission is difficult to determine even when using WGS data (Rodríguez-Prieto et al. 2012; Biek et al. 2012; Crispell et al. 2017).

Another advantage of WGS is its ability to add a time frame that allows for the estimation of when an event took place, compared to other genotyping techniques, although larger sample sizes are needed for accuracy (Hlokwe et al. 2016; Crispell et al. 2017). The ability of molecular epidemiology to determine when isolates diverged increases our insight into *M. bovis* infections, therefore helping to identify the most likely source of infection with higher confidence (Crispell et al. 2017).

The high diversity of *M. bovis* isolates in MP is somewhat surprising since the park is less than 18km$^2$ and geographically isolated only by the Crocodile River from KNP. In contrast, KNP has larger free-roaming populations of wildlife, but the genetic diversity of *M. bovis* appears to be less. A reason for the low genetic diversity could be a potential bottleneck

resulting in a founder effect, leading to a single strain or strains that share a closely related clonal complex becoming dominant (Smith et al. 2006). However, the high genetic diversity from MP needs to be further investigated as the variation in such a small population was expected to be less than in larger populations, unless there were repeated introductions of *M. bovis* into MP (Smith et al. 2006).

This study was limited by the low number of isolates available for WGS, which restricted the analysis. A further limitation was that we had no data regarding contact between animals in MP. Future studies should investigate the use of specific diverging markers that may help to further characterise specific strains and allow rapid genotyping. Additional samples from the GKNP will enable analysis using a molecular clock which could determine the approximate time that *M. bovis* was introduced to KNP and MP.

To conclude, the study determined that *M. bovis* isolates from MP and KNP form two distinct clades. However, these isolates would have been regarded as the same if only spoligotyping and RD-analysis (RDbovis(c)_Kruger) were used for genotyping. Furthermore, phylogenetic analysis confirmed that isolates from MP share a common ancestor with isolates from KNP. Additionally, the diversity of MP isolates was confirmed by the WGS analysis and showed that these were genetically more diverse than isolates from KNP.

**Conflict of interest**

None to declare.

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# Chapter 8

## General Discussion and Conclusion

**Eduard O. Roos**

**My contribution to this chapter:** Writing of chapter

*Mycobacterium bovis* is endemic in regions of South Africa, including in the Greater Kruger National Park (GKNP), where abundant warthog populations are found. This study is the first to document the high prevalence of culture-confirmed *M. bovis*-infection in warthogs in endemic regions of South Africa. Animals from the GKNP were infected with strains belonging to two distinct clades but with notable genetic diversity. Importantly, three serological assays, evaluated for the first time in this species, displayed high sensitivity and specificity in detecting infected individuals. Moreover, assays of cell-mediated immunity, measuring antigen-induced *CXCL10* transcription and IP-10 production in whole blood, showed promise as tests of *M. bovis* infection.

Assays which measure serological responses in warthogs match the sensitivity and specificity reported in studies on other suids (Aurtenetxe et al. 2008; Boadella et al. 2011; Cardoso-Toset et al. 2017). Furthermore, warthogs develop an early, strong and measurable humoral response to *M. bovis*. Therefore, these assays facilitate serosurveillance studies, providing an overview of infection on a population level, as well as provide information on individual infection status. For population level screening, the indirect PPD ELISA is the optimal assay as many samples can be run simultaneously (Boadella et al. 2011). Alternatively, for individual or pen-side testing, i.e. testing warthogs in zoos, the DPP® VetTB is ideal as it delivers a result within 20 min (Boadella et al. 2011). These diagnostic tools allow for easy blood-based testing of warthogs.

The seroprevalence results, of warthogs in *M. bovis* endemic locations, closely matched the prevalence based on mycobacterial culture. This was unexpected as previous research suggested that warthogs are mainly a dead-end host and only reported prevalence of 0-2% in warthogs, therefore their ecological consequences of infection were not considered

(Woodford 1982; Tschopp et al. 2010). However, the prevalence in this study was similar to that found in feral suids, a maintenance host in the Mediterranean ecosystem (Vicente et al. 2007; Naranjo et al. 2008; Santos et al. 2009). The high *M. bovis* infection rate supports the usefulness of warthogs as disease sentinels, since ideal sentinels should be abundant and highly susceptible (Nugent et al. 2002). Yet the role warthogs play in the epidemiology of bovine tuberculosis (bTB) has been neglected in the past and should receive more attention. It is also unknown if this disease could be a significant concern for the animals themselves, transmission to other species, especially predators, or as a threat to public health when consumed as bushmeat. This emphasises the importance of including warthogs when studying the epidemiology of bTB in African ecosystems.

In contrast, an assay measuring antigen-specific cell-mediated immune (CMI) responses, the interferon gamma (IFN-γ) release assay, was not consistent in this species. However, interferon gamma induced protein 10 (IP-10), and the gene coding for this protein, *CXCL10*, showed promise as sensitive and specific biomarkers of infection. These findings highlight the role that these genes and their proteins (i.e. IP-10) may play as potential biomarkers of bTB in wildlife (Goosen et al. 2014; Olivier et al. 2015; Higgitt et al. 2017). Furthermore, the high sequence homology between warthogs and domestic pigs suggests the potential to use this gene expression assay in other suids, enabling the measurement of an antigen-specific CMI responses.

The cytokine IP-10 was identified as a useful biomarker of antigen-specific immune activation in warthogs, although IFN-γ could not be reliably measured. Measures of CMI responses are important to understand the immunobiology of the host to *M. bovis* infection. There are only a few reports of cytokine release assays in other suids, with regards to *M.*

*bovis* infection (Pesciaroli et al. 2012, Dr Julio Alvarez pers. comm. 2016). Even though the sensitivity and specificity of the IP-10 assay are less than for the serological assays, IP-10 is still a promising biomarker for detection of *M. bovis*-infected warthogs. This cytokine has also been reported to be a sensitive and stable biomarker for the identification of *M. bovis*-infected African buffalo (Goosen et al. 2015, 2016). This approach can also be useful to investigate the CMI response in other wild suids, since few studies have been able to measure cytokines such as IFN-γ, in suids infected with *M. bovis*.

Whole genome sequencing (WGS) of *M. bovis* strains isolated from infected warthogs demonstrated that these suids are commonly infected with the *M. bovis* strain which is dominant in the GKNP; however, high genetic diversity was observed between isolates. The spoligotype and WGS diversity is indicative of ongoing transmission (Duarte et al. 2008). A clonal expansion of *M. bovis* is seen in KNP, although the high diversity of *M. bovis* outside KNP would suggest that the bacteria is undergoing divergence (Smith et al. 2006). Furthermore, two distinct clades were identified with the same spoligotype strain, supporting the divergence hypothesis. This challenges the current thought that the SB0121 spoligotype, dominant in Kruger National Park (KNP), is the cause of infection in animals within the GKNP, as suggested previously (Hlokwe et al. 2014). It is therefore likely that a geographical barrier, like a river, can prevent the transmission and reintroduction of *M. bovis* across these borders.

In conclusion, this PhD described the development and use serological and CMI response (tuberculin skin test, GEA and cytokine release) assays, all which could identify and distinguish between *M. bovis*-infected and uninfected warthogs. Therefore, creating a platform for researchers to study the immunology of warthogs with regards to bTB. The

unexpected high prevalence of four warthog populations, from within bTB endemic regions, were described and highlighted that warthogs are highly susceptible to *M. bovis* and the rate of infection is more frequent that originally thought. Lastly, this PhD identified that there is a high genetic diversity among *M. bovis* isolates from the south of GKNP, which is geographically a relatively small space. This PhD provides evidence that warthogs, in endemic areas, are good sentinels and may play a previously unrecognized role in bTB epidemiology in African ecosystems.

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