

## Chapter 5

### Discussion and concluding remarks

#### 5.1 Introduction

In many countries, despite the enormous expenditure on programmes for the control and eradication of bovine tuberculosis (BTB), it remains a serious problem (Pollock & Neill 2002), because of the persistence of infected wildlife reservoirs that have contact with cattle (Schmitt, Fitzgerald, Cooley *et al.* 1997; Corner, Stevenson, Collins & Morris 2003; Renwick, White & Bengis 2007). In reservoir hosts the infection persists through horizontal transfer in the absence of other sources of *Mycobacterium bovis*. Depending on the prevalence and the susceptibility of a species to a *M. bovis* infection, *M. bovis* may be transmitted to spill-over hosts such as chacma baboons (*Papio ursinus*), lions (*Panthera leo*), cheetahs (*Acinonyx jubatus*) and leopards (*Panthera pardus*) (Michel, Bengis, Keet *et al.* 2006; Etter, Donado, Jori *et al.* 2006). In spill-over hosts, except in primates, infections may occur sporadically or persist within these populations if a true maintenance host is present in the ecosystem (Buddle, Skinner & Chambers 2000; Etter *et al.* 2006). In the South African context the overall prevalence in cattle is low but it does occur in all provinces. However, in wildlife it is restricted to a few reserves where it occurs at high prevalence rates (Michel, Hlokwe, Coetzee *et al.* 2008).

*Mycobacterium tuberculosis* infections have mostly been reported in Asian elephants (*Elephas maximus*) (Mikota, Miller, Dumonceaux *et al.* 2008). It is estimated that countries in the South-East Asian Region have a 36% incidence of human TB (WHO 2008). This high prevalence makes it likely that many elephants within Asia are also infected. India is reported to have the largest burden of human TB with an estimated incidence of 1.8 million new cases annually (Steinbrook 2007; WHO 2008) and it is also home to half of all wild Asian elephants along with approximately 3 500 captive elephants (Vidya, Fernando, Melnick & Sukumar 2005; Hammatt 2007). Not only do infected humans pose a threat to these valuable animals but *M. tuberculosis*-infected elephants are potential reservoirs that could infect wildlife and humans who may come in close contact with them (Michalak, Austin, Diesel *et al.* 1998). The improper management of TB in humans is the major cause of emerging multi-drug resistant-TB (MDR-TB). Herein lies the concern for the transmission of MDR-TB from infected humans to elephants. One case has already occurred in the USA (Lyashchenko,

Greenwald, Esfandiari *et al.* 2006). No cases have been reported in Asia yet. If a new form of MDR-TB evolves in elephants it could be fatal to humans as well as elephants that may already be resistant to currently used drugs (Hammatt 2007).

## 5.2 Diagnosis

*Ante-mortem* detection of *M. bovis* and *M. tuberculosis* infections in wildlife is difficult because of the delayed appearance of clinical signs (Ashford, Whitney, Raghunathan & Cosivi 2001) and deficiencies in available diagnostic tests (de Lisle, Bengis, Schmitt & O'Brien 2002). Interferon-gamma (IFN- $\gamma$ ) tests are *in vitro* cell mediated assays that monitor cell responses to specific antigens, such as purified protein derivatives (PPDs) by the detection of IFN- $\gamma$  produced. These *in vitro* blood tests provide a distinct advantage especially with wild animals as they are only confined once (Cousins 2008). Even in humans for whom the Mantoux reaction is well established worldwide, there is a strong tendency for its replacement by the IFN- $\gamma$  assay (Pottumarthy, Morris, Harrison & Wells 1999; Herrmann, Simonney & Lagrange 2007). In children these IFN- $\gamma$  tests, which make use of both the early secretory antigenic target (ESAT-6) and culture filtrate (CFP-10) proteins, are easier to perform and have a greater specificity, especially since they do not cross-react with the Bacillus Calmette-Guérin (BCG) vaccine and most other environmental bacteria (Blanc, Dubus, Garnier *et al.* 2008).

## 5.3 Control measures

In the African context, since no cases of *M. bovis* and / or *M. tuberculosis* infections have been reported in free-ranging or captive species of rhinoceros and African elephants (*Loxodonta africanum*), the underlying issue for diagnosis is early detection because the goal is to keep the population free of infection. In captive situations, if mycobacterial infections are not diagnosed in these animals there is the risk of infection to other rhinoceroses, elephants, and other species of animals, as well as to caretakers and visitors (Dalovisio, Stetter & Mikota-Wells 1992; Lyashchenko *et al.* 2006).

It is known that elephants can show no signs of clinical disease but may shed the organism (Lyashchenko *et al.* 2006). If these animals are diagnosed early in the course of the infection they can be isolated and specifically treated with antibiotics thus reducing the risk of

exposure to other zoo animals, handlers and visitors to the exhibits. Guidelines and protocols for the treatment of elephants are defined in the *Guidelines for the Control of Tuberculosis in Elephants* <http://www.elephantcare.org/protodoc> (Mikota *et al.* 2008). Starting treatment at the earliest stages of disease, before shedding, could potentially lead to improved cure rates, a decreased likelihood of the development of MDR-TB strains and reduced transmission of infection (Lyashchenko *et al.* 2006). Treatment of infected elephants with antibiotics (Maslow, Mikota, Zhu *et al.* 2005; Peloquin, Maslow, Mikota *et al.* 2006) is an expensive procedure as drugs may cost up to \$50 000 to treat one elephant for one year (Hammatt 2007). The possibility of euthanasia should be considered for those animals found to have MDR-TB as they would pose a greater risk to other animals and humans with whom they come in contact if not identified and quarantined (Hammatt 2007).

Identification of antigens for use in an IFN- $\gamma$  ELISA for the detection of infectious animals, which present a high risk of contamination to humans, elephants and other mammals is required (Greenwald, Lyashchenko, Esfandiari *et al.* 2009). Currently, serological tests for TB diagnostics in elephants are based on antigen cocktails, which usually detect animals in the active or advanced stages of the disease (Lyashchenko *et al.* 2006). In many of these cases the host is already exhibiting terminal disease and serology-based tests therefore do not play a significant role in defining infected hosts during the early stages of infection and have not yet proven to identify shedders. In humans serology-based tests have no value in identifying TB and it is for this reason that efforts are being put in place for IFN- $\gamma$  testing by searching for appropriate antigens (Steingart, Henry, Laal *et al.* 2007). This can be achieved by testing antigens or combinations of antigens as has been done in cattle by differentiating between infected and vaccinated animals (DIVA strategy) (Rhodes, Gavier-Widen, Buddle *et al.* 2000b; Vordermeier, Whelan, Cockle *et al.* 2001; Koo, Park, Ahn *et al.* 2005). Antigens, such as, MPB70, MPB83 and PPDs (Rhodes, Buddle, Hewinson & Vordermeier 2000a) can be used for early detection. Immuno-dominant antigens ESAT-6 (Pollock & Andersen 1997) and CFP-10 can be used for the detection of active disease (Rhodes *et al.* 2000a; Koo *et al.* 2005). The IFN- $\gamma$  tests established in this thesis can make use of such antigens to identify animals excreting the infectious organisms and thus posing an immediate threat to other animals.

Studies leading towards the development of real-time reverse transcription-PCR assays to quantify IFN- $\gamma$  in many wildlife species (Harrington, Surujballi, Waters & Prescott 2007) and other cytokines, such as TNF- $\alpha$ , IL-2, IL-10 and IL-12 in Asian elephants (Landolfi, Schultz, Mikota & Terio 2009), show improvements to diagnostic techniques targeting CMI response. These tests may assist in monitoring the prevalence of disease in various wildlife populations and can be used as screening tools for animals entering disease-free zones or herds (Harrington *et al.* 2007).

#### 5.4 Development of an IFN- $\gamma$ enzyme-linked immunosorbent assay (ELISA)

The present study demonstrates the use of different techniques towards the production of antibodies for the development of IFN- $\gamma$  ELISAs for the detection of *M. bovis* and *M. tuberculosis* infections in rhinoceroses and elephants. *Mycobacterium bovis* infections are a major problem in wildlife species and the detection of pro-inflammatory cytokines (IFN- $\gamma$ , TNF- $\alpha$ ) have been used to identify animals during the early stages of infection. To date IFN- $\gamma$  assays have been developed for cattle (Rothel, Jones, Corner *et al.* 1990), humans (Desem & Jones 1998) non-human primates (Vervenne, Jones, van Soolingen *et al.* 2004), cervids (Waters, Palmer, Thacker *et al.* 2008), badgers (Dalley, Davé, Lesellier *et al.* 2008) and cats (Rhodes, Gruffydd-Jones, Gunn-Moore & Jahans 2008).

For such a test to be developed and validated for rhinoceroses and elephants, much research is yet to be performed in order to define antigens which could be chosen according to the epidemiological situation and used to stimulate PBMCs and / or blood. The primary approach for rhinoceroses and African elephants in wildlife conservation areas is to provide an additional laboratory-based guarantee that documents these animals as free of *M. bovis* and *M. tuberculosis* infections. Under these conditions, it is equally important to detect infection as early as possible in these populations, in order to avoid the spread to congeners which would jeopardize any further translocation of these valuable animals, and thus putting all the conservation efforts at risk. In view of the prevalence of MTBC infection in Asian elephants the approach would lean towards detecting active disease with the use of antigens such as ESAT-6 and CFP-10. For animals in zoo collections and in circuses, the first step would be to guarantee that animals are not infected. In case of infection, it will become critical to demonstrate that animals are not contagious and to monitor this over time and / or to follow the efficacy of treatment.

A major part of this study was the production of both monoclonal and polyclonal antibodies which were used to develop different ELISA formats to detect recombinant RhIFN- $\gamma$  and AsEpIFN- $\gamma$ . The hybridoma technique was used to generate murine monoclonal antibodies against rRhIFN- $\gamma$  (Chapter 2) and rAsEpIFN- $\gamma$  (Chapter 4). Chickens were used for the production of polyclonal antibodies (Chicken IgY; Chapter 2). In Chapter 3 the phage-display technique was applied to generate chicken antibodies against recombinant RhIFN- $\gamma$  and AsIFN- $\gamma$ .

For production of polyclonal antibodies, chicken housing proved to be inexpensive, egg collection non-invasive, and the isolation and affinity purification of IgY antibodies showed to be fast and simple. In addition chicken IgY was used successfully in ELISA formats where it was used as a detecting antibody. Monoclonal antibody production proved to be a more expensive technique, was time consuming and required a higher level of skills to be performed. Despite these drawbacks once made they served as a constant and renewable source of antibody.

In Chapter 2 only one monoclonal antibody, 1H11, proved to be suitable for the capture of rRhIFN- $\gamma$  using polyclonal IgY<sup>uu</sup> as detecting antibody. The capture ELISA was used to determine whether both the recombinant and native forms of white RhIFN- $\gamma$  could be detected. Results indicated that both forms of the IFN- $\gamma$  can be detected in this format of the ELISA. Although the applicability of IFN- $\gamma$  ELISA has been shown for native white RhIFN- $\gamma$  this is not yet the case for black rhinoceroses (*Diceros bicornis*). In addition, the detection limits, sensitivity and the specificity of the test has yet to be determined in both species of rhinoceroses, and conditions such as non-specific sensitization caused by other mycobacteria potentially influencing the test outcome need to be investigated.

When the clone 1H11 was no longer secreting antibody the switch to the subclone 1D11 was made for further secretion of the required antibody. This antibody was used as a detecting antibody in ELISA formats using the single chain variable fragment (scFv) as the capture antibody (Chapter 3). Since monoclonal antibodies only recognize one epitope on any one antigen they are less likely to cross-react with other proteins and because of their specificity they are excellent as a primary antibody in an assay and will give significantly less background than polyclonal antibodies. Using the scFv and 1D11 combination (Chapter 3)

proved successful for the detection of rRhIFN- $\gamma$ , but not for rAsEpIFN- $\gamma$  indicating specificity of antibody 1D11 towards the rRhIFN- $\gamma$ . When 1D11 was replaced with IgY as a detecting antibody in the scFv ELISA format, this polyclonal antibody detected both rRhIFN- $\gamma$  and rAsIFN- $\gamma$ , however background did increase.

In Chapter 4 the combination of monoclonal antibodies 21 and 5 for capture and detection of rAsEpIFN- $\gamma$  worked well. Since it is preferable to include a monoclonal pair for the IFN- $\gamma$  ELISA, this combination along with the 1F5/1D11 combination mentioned above, provided an ideal result as these antibodies react with different epitopes on the same antigen. Studies conducted by Dalley *et al.* (2008) indicated that IFN- $\gamma$  ELISAs based on monoclonal antibody pairs were more sensitive than using polyclonal serum. In addition, they mention that it is more sustainable compared to a finite supply of polyclonal reagents. Bovigam<sup>®</sup> (Wood & Jones 2001), the human gamma-interferon assay (Desem & Jones 1998) and the gamma-interferon assay for badgers (Dalley *et al.* 2008) make use of monoclonal antibody pairs for the capture and detection of IFN- $\gamma$ .

Initially the phage-display approach proved to be rather disappointing, as attempts at finding clones using the naïve semi-synthetic Nkuku<sup>®</sup> library (van Wyngaardt, Malatji, Mashau *et al.* 2004) proved unsuccessful. Various bio-panning steps were performed and each panning resulted in no enrichment. This could mean that none of the antibodies displayed in this library had an affinity towards the recombinant antigens. Consequently a phage-display immune library targeted against rRhIFN- $\gamma$  was constructed which, like the hybridoma technique, necessitated the immunization of an animal. Chickens were used as a source of immunoglobulin genes to construct the antibody phage library. Previous studies (Davies, Smith, Birkett *et al.* 1995; Yamanaka, Inoue & Ikeda-Tanaka 1996; Wyngaardt *et al.* 2004) report the successful production of chicken recombinant scFvs by phage display.

Another difference between the traditional hybridoma technique and the phage-display method of generating antibodies in this study was the use of chickens instead of mice, although mice (Okamoto, Mukai, Yoshioka *et al.* 2004) have also been used to construct scFv phage-display libraries. Unlike mice, chickens have only single functional V and J segments for the heavy and light chain loci (McCormack, Tjoelker & Thompson 1991). The genes formed by these rearrangements are diversified in the bursa of Fabricius by gene conversion.

This process introduces long tracts of nucleotides from upstream V pseudogene segments into the rearranged V regions by nonreciprocal homologous recombination (Reynaud, Anquez, Grimal & Weill 1987; Thompson & Neiman 1987; Reynaud, Dahan, Anquez & Weill 1989).

Immune libraries are different from naïve semi-synthetic libraries in that they are smaller in size and therefore diversity is far less (Burton 1995) because the amplified V-region genes will already have undergone affinity maturation and clonal selection *in vivo*. However, an immune library would be biased towards the immunizing antigen and the probability of isolating binders would be much higher than using a naïve and or a semi-synthetic library in this situation. Good quality antibody phage libraries are reliable sources of useful antibodies when panned against purified antigens (Winter, Griffiths, Hawkins & Hoogenboom 1994) or even complex antigen mixtures (Marks, Ouwehand, Bye *et al.* 1993)

Since the rhinoceros and the elephant IFN- $\gamma$  genes share a high nucleotide (nt) and amino acid (aa) similarity (Fig. 3.1) it provided the opportunity to pan the RhIFN- $\gamma$  library against recombinant Asian elephant IFN- $\gamma$  (rAsEpIFN- $\gamma$ ) assuming that possible binders against this antigen could also be recovered. After initial panning the polyclonal phage ELISA indicated that there was enrichment of specific binders to both the recombinant antigens (rRhIFN- $\gamma$  and rAsEpIFN- $\gamma$ ). Single colonies were selected for screening in a monoclonal phage ELISA and binders were selected against each of the recombinant antigens. Selected clones were sequenced and showed that clones against rRhIFN- $\gamma$  shared the same sequence. Similarly the clones selected against rAsEpIFN- $\gamma$  showed identical sequences. The sequence of the clone against rRhIFN- $\gamma$  differed when compared to the sequence of the clone identified against rAsEpIFN- $\gamma$ . Direct ELISAs that were performed showed cross-reactivity between the anti-rRhIFN- $\gamma$  and AsEpIFN- $\gamma$  binders. The two clones (1F5 & 6D7) of different sequences, which were identified in Chapter 3, showed a higher affinity towards rRhIFN- $\gamma$ . Although clone 1F5 showed an affinity to rAsEpIFN- $\gamma$ , it showed a much stronger affinity to rRhIFN- $\gamma$ . This point emphasizes the fact that in order to get high affinity antibodies to a specific antigen, the animal should be immunized by that antigen to elicit an immune response. By immunizing with rRhIFN- $\gamma$  the response was restricted to that antigen and therefore both scFvs (1F5 & 6D7) showed a greater affinity to rRhIFN- $\gamma$  than to rAsEpIFN- $\gamma$ .

The end result was disappointing as only eight clones were found and only two clones did not share the same sequence. Baek, Suk, Kim & Cha (2002) mention that phagemid vector systems have very low display levels of antibody:pIII fusions, and that the possibility of useful antibodies can be missed during the selection process. Since this library was based on the above-mentioned vector system with the scFv inserted within pIII region could be a possible explanation why only two clones were isolated. Another limitation during the isolation of specific binders from the RhIFN- $\gamma$  phage-display library was the concentration of the panning antigen. A concentration of 10  $\mu\text{g/ml}$  was used during all rounds of bio-panning but only half this concentration was used during the screening. In addition, the washing stringency was maintained during all rounds of bio-panning. Selection of high affinity antibodies is favoured by increasing stringency in the washing steps after the first round and by reducing the antigen concentration during subsequent rounds of panning (Bradbury and Marks 2004).

Selection which involves two antibodies with different affinities will favour the antibody with the higher affinity. Two factors that adversely affect the selection efficiency are toxicity to the bacterial host and display levels (function of antibody folding efficiency, susceptibility to proteolysis and tendency to aggregate). It is for these reasons that if selections are continued for many rounds, diversity usually becomes reduced to a single clone (Bradbury and Marks 2004). It may be therefore better to assess diversity earlier rather (after round two or three) than later as diversity may be reduced with more bio-panning steps. Furthermore the result also raises questions regarding the quality of the immune library that was constructed. It could mean that the immune library was of a poor quality since it failed to yield a larger number of clones. It is worth noting that less than a 100 clones were screened. If more clones were screened the possibility of finding a greater number of clones could have increased.

Although only two antibodies were isolated using the phage-display approach, it did prove to be advantageous as it was cost effective and not as time consuming as the conventional hybridoma method. The antibodies produced have the potential to be manipulated to improve their specificities for use in diagnostic tests. These antibodies were rapidly derived and characterized using standard molecular techniques. With the hybridoma approach whole antibodies (IgG molecules) were derived whereas with the phage-display library approach fragments of antibodies (scFvs) were derived and thus could be expressed in *E. coli*. The



scFv format is better tolerated by bacteria, less likely to be degraded, can form dimers (diabodies), is a single protein molecule and the (Gly<sub>4</sub>Ser)<sub>3</sub> linker can be shortened. However, they are less stable than IgG molecules, and a fraction of expressed scFv can be non-functional (Bradbury & Marks 2004). The scFvs isolated in this study have to be tested for stability since they are going to be used as potential diagnostic reagents. This will be performed as outlined by Chiliza, van Wyngaardt & du Plessis (2008).

The detection limits of the capture ELISAs developed in this study do not compare favourably with those already established for commercial cattle and primate IFN- $\gamma$  capture ELISAs, and thus need to be optimised further. However, they do provide a first test to optimise the ELISA for the detection of native IFN- $\gamma$  of Asian elephants. Since the AfEpIFN- $\gamma$  gene has been shown to share a high homology on both the nt and aa level (Chapter 4) a single test would most likely serve for the detection of mycobacterial infections in both species. In addition, scFv antibodies will be tested in combination with those antibodies produced against rAsEpIFN- $\gamma$  (Chapter 4) in order to improve the analytical and diagnostic sensitivity of these IFN- $\gamma$  tests. Possible ways of increasing the sensitivity of the ELISAs include the incubation of the various steps at room temperature and comparison with that of ELISAs performed at 37°C, increasing the stringency of the wash steps, conjugation of the detecting antibodies with HRP and also substitution of the substrate, OPD, with tetramethylbenzidine (TMB) substrate. The latter has been used in IFN- $\gamma$  detection ELISAs for cattle (Rothel *et al.* 1990), humans (Desem & Jones 1998) and badgers (Dalley *et al.* 2008).

The development of the IFN- $\gamma$  assay may be critical in antigen discovery. It is important to re-emphasise that different antigens may be used for different purposes (the OIE “fit for purpose” strategy), and thus be utilized as a tool for differentiating infected, infectious, vaccinated and non-vaccinated animals as well as latent carriers. Furthermore by identifying target antigens and using them to determine correlates of disease severity, the assay could help in predicting the success or failure of vaccination studies (Hope & Villarreal-Ramos 2007) and of antibiotic treatment.

The field application of the IFN- $\gamma$  test in wildlife settings will prove challenging as blood samples have to be processed within 8 hours of collection (Rothel *et al.* 1990; Schiller,

Waters, Vordermeier *et al.* 2009). In addition, other factors such as pre- and post-collection parameters will affect the accuracy of the IFN- $\gamma$  test in field scenarios. Schiller *et al.* (2009) address such concerns regarding the whole blood IFN- $\gamma$  assay in cattle. These findings, although established for cattle, will assist in improving the IFN- $\gamma$  tests developed in the present study and further work linked to the validation of these ELISAs.

## 5.5 Conclusion

The overall endeavour of this study was to make use of molecular- and immunology-based techniques to develop a capture ELISA for the detection of IFN- $\gamma$  in wildlife species, such as rhinoceroses and elephants. The diagnostic tools developed in the study have resulted in the set up of various capture ELISAs that will contribute to the establishment of *ante-mortem* diagnostic tests for the detection of *M. bovis* and *M. tuberculosis* infections in both rhinoceroses and elephants. These tools may be used in parallel and complementary to existing serological tests and thus contribute to the management and control of mycobacterial infections in these four species. It is anticipated that the IFN- $\gamma$  test for TB / BTB diagnostics for rhinoceroses and elephants will prove to be advantageous in detecting not only infected animals but also infectious animals (shedders). Future research requires the detection of native IFN- $\gamma$  of both rhinoceroses and elephants, using the tools generated in this study and the identification of antigens that can be used in the IFN- $\gamma$  ELISA for the detection of infectious animals before the onset of clinical signs. Once this is established, steps can be taken to validate the diagnostic test with samples of known TB and BTB positive animals, and subsequently the sensitivity and specificity of the test may be determined. When this is achieved it will provide a management tool for not only screening, detection and diagnosis of *M. bovis* and *M. tuberculosis* infections in rhinoceroses and elephants, and also the identification of infectious animals.