Interferon-gamma ELISA's for the detection of *Mycobacterium bovis* infections in lions (*Panthera leo*)

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Interferon-gamma ELISA's for the detection of *Mycobacterium bovis* Infections in lions (*Panthera leo*)

Research Project Veterinary Medicine University Utrecht

by

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January - March 2010

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Abstract

In this research project steps toward the development of an IFN-γ ELISA for the diagnosis of bovine tuberculosis in lions are made. Two different existing IFN-γ ELISA's were studied and tested. An ELISA, previously developed for the detection of recombinant rhinoceros IFN-γ (Morar *et al*. 2007), was used to determine detection of recombinant feline IFN-γ. An adapted feline IFN-γ ELISA, developed by Rhodes *et al*. (2008) was the other ELISA system that was used in this research. Blood samples from both domestic cats and lions were collected and processed for use in the two different ELISAs that are mentioned above. Inconclusive results were obtained during this study, however this report describes the various steps that were performed and results obtained during a research period of three months.

1. Introduction

1.1 *Mycobacterium bovis*

Mycobacteria belong to the family Mycobacteriaceae. Many species of the Mycobacteriaceae family are harmless environmental *Mycobacteria*. However, some members of this family belong to the *Mycobacterium tuberculosis* complex (MTBC) and are known to cause tuberculosis (TB) in both humans and animals (Table 1).

Table 1: Members of the MTBC (Engleberg *et al.* 2007)

***Mycobacterium* Species**	**Host**
M. tuberculosis	Humans
M. bovis	Livestock and Wildlife
M. africanum	Humans
M. microti	Rodents
M. pinnipedii	Seals

Mycobacterium bovis is an acid-fast, rod shaped and slightly curved anaerobic, non-motile and non spore forming bacillus (Quinn *et al.* 2002) and is the causative agent of bovine tuberculosis (BTB) in cattle and many wildlife species (Michel *et al.* 2006).

1.2 *Mycobacterium bovis* in the Kruger National Park (KNP)

Bovine tuberculosis (BTB) is a problem of great importance in the Kruger National Park (KNP), South Africa (Michel *et al.* 2006). The disease probably originated from cattle, imported from Europe during the 18th and 19th century and initially affected African buffaloes (*Syncerus caffer*) (Keet *et al.* 2000, Michel *et al*. 2006). In the KNP BTB was first diagnosed in a buffalo in the year 1990. At this time the disease was only seen in buffaloes in the southern part of the park (Bengis *et al.* 1996, Keet *et al.* 2000, de Vos *et al.* 2001). Since then the disease has been spreading to the north of the park and the number of infected herds is still growing (Keet *et al.* 2000). African buffaloes are considered to be the main reservoir hosts for *M. bovis* infections in the KNP (Michel *et al.* 2006).

The lions (*Panthera leo*) in the KNP have also been affected with BTB (Keet *et al.* 1996). As in buffalo, the prevalence of BTB is the highest in the southern part of the park (Keet *et al.* 2000, Michel *et al*. 2006). It always was suspected that lions got infected with *M. bovis* by eating infected buffalo, one of their preferential prey species (Keet *et al.* 2000). African buffalo which are infected with *M. bovis* are likely to be the weakest animals in a herd, and hence the first to be killed by predators like lions (Keet *et al.* 2000). When lions ingest infected meat they can get infected with *M. bovis*. The lions could also get infected with *M. bovis* by aerosols when they perforate the trachea of the prey with their canines in the suffocating process (Keet *et al.* 2000). Recent results, of genetic typing of *M. bovis* isolates that were obtained from infected tissues of lions and buffaloes indicate that the lions do get infected via ingestion of infected buffalo tissue (Michel *et al.* 2009).

Lions are now considered to be able to maintain the *M. bovis* infection in their population, making them a maintenance host, instead of a spillover host (Keet *et al.* 2000*,* Maas 2008). The main route of infection between lions is thought to be through aerosols, but it could also be transferred through biting wounds and milk (Keet *et al.* 2000*,* Maas 2008). Lions which are infected with *M. bovis* tend to die at a younger age than uninfected individuals, and have symptoms like depression, emaciation, unilateral ocular lesions, swollen joints, elbow hygromas, alopecia and severe dyspnoea (Maas 2008).

The spread of the disease is significant, especially since the KNP is being expanded by fusing with a combination of several other parks (the Limpopo National Park in Mozambique and the Gonarezhou National Park, Manjinji Pan Sanctuary and Malipati Safari area in Zimbabwe). This will be known as 'The Great Limpopo Transfrontier Park' (Figure 1.1) (www.greatlimpopopark.com). The formation of this new park could increase the spread of *M. bovis* to the other areas that will be linked to the KNP. There is also the potential that infected animals are a danger to livestock and people on the farms near the park (Michel 2002; Maas 2008). It is therefore important to determine the BTB status of the lion population in the KNP and the development of a reliable test would be a step in the right direction in achieving this aim.

Figure 1.1 Map of The Great Limpopo Transfrontier Park. It contains the Kruger, Limpopo, Gonerezhou, Banhine and Zinave National Park (indicated in Green). (www.greatlimpopopark.com)

1.3 Diagnostic tests

Diagnostics tests that are currently used include the detection of acid-fast bacilli in histopathology in combination with a positive culture from fresh biopsy material. This is regarded as the gold standard (Keet *et al.* 2010*)*. Due to the slow growth rate of *Mycobacteria* on culture, it may take between six to eight weeks before results are obtained.

After culturing a *Mycobacterium* species, the polymerase chain reaction (PCR) technique can be used to determine if the *Mycobacterium* that is growing on the culture plate is a member of the *M. tuberculosis* complex or not (Keet *et al.* 2010, Maas, 2008). In this technique a specific segment of DNA from *M. bovis* is amplified. The PCR technique can also be used to determine the presence of *M. bovis* from saliva and other excreta or infected tissues. In a comparison study involving several laboratories, the outcome indicated big differences between the laboratories in rates of false positive results and levels of sensitivity (Noordhoek *et al.* 1994). Due to this PCR technique is thought to be unreliable for use as a diagnostic system for *M. bovis* infections. More recently, a new interest in PCR diagnostics for TB in humans has been initiated, but a good working test has yet to be developed (Gupto *et al.* 2010, Sankar *et al.* 2011).

Serology-based tests can also be used for the detection of infection with *M. bovis*. These tests can detect antibodies against *M. bovis.* Serology seems to be related to lesions and shedding of *M. bovis*. If control of the disease is the aim than the detection of shedders with serology based tests is a good option. If the aim is to detect early infections to be able to remove infected animals that appear healthy from the pride other tests have to be used.

The most useful diagnostic test that is currently used in lions for the detection *M. bovis* infections is the intradermal administered tuberculin skin test (IDT). This test has been validated for use in lions (Keet *et al*. 2010). To distinguish between *M. bovis* and environmental mycobacterial exposure specific proteins derived from *M. avium* (PPDA) and *M. bovis* (PPDB) are used in the skin tests. Most of the lions that were positive in culture showed a distinct response to PPDB and responded variable and less distinct on PPDA. Lions from *M. bovis* free areas did not respond to the bovine tuberculin PPD. The test had a sensitivity of 80.8 – 86.5%, when the bovine tuberculin PPD test alone was considered. The disadvantage of the IDT is that the animal has to be captured twice which is not an easy objective to accomplish if you are handling wild animals.

An alternative test, which could also be used in parallel with the IDT, is the IFN-γ assay. The IDT and the IFN-γ assay both detect the presence of IFN-γ. The former is performed *in-vivo* and the latter is an *in-vitro* form of the IDT (Figure 1.2) (Andersen *et al.* 2000). In this case recapture of the lions is unnecessary and it has already proven to be reliable and very successful in diagnosing BTB in cattle (de la Rua-Domenech *et al.* 2006).

This test can also distinguish between pathogenic and more harmless environmental *Mycobacteria*. For this aim again PPDA and PPDB can be used. Research has been done to find more antigens for this purpose and to improve the reliability of the test. Vordemeier *et al*. (2001) describes the potential of two antigens, culture filtrate protein 10 (CFP10) and early secretory antigenic target 6 (ESAT-6). The genetic information that codes for these antigens is present in the genome of members of the *M. tuberculosis* complex but deleted in the genome of most environmental *Mycobacteria* (Maas 2008). There also is a hybrid of these antigens, CFP10-ESAT-6, that can also be used in tests. ESAT-6 is known to give a positive test in most humans with *M. tuberculosis* infection. The immune response to the antigen correlates with the progression and severity of the disease (Anderson *et al.* 2000, Demissie *et al.* 2006).

Another antigen that can be used to differentiated between *M. bovis* infection and infection with environmental *Mycobacteria* is purified protein derivate Fortuitum (PPDF). The possibilities of using this antigen derived from environmental *Mycobacterium fortuitum* in an IFN-γ ELISA for *M. bovis* detection in buffalo have been explored by Michel *et al.* (2008) and Michel 2008.

The lion IFN-γ gene was sequenced by Maas *et al*. (2010) so there is a basis to develop recombinant lion IFN-γ and a specific lion IFN-γ specific ELISA for the detection of *M. bovis* infections in lions. Rhodes *et al.* (2008) adapted the cat IFN-γ assay and reported that it may be used for the detection of IFN-γ from lions.

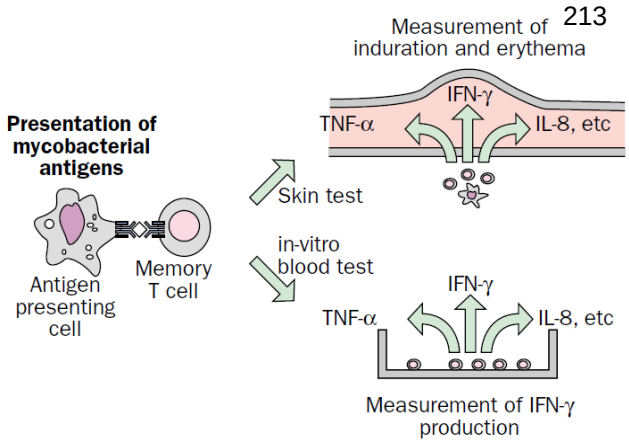

Figure 1.2 The *in-vivo* and *in-vitro* mechanisms of the IDT (Andersen *et al.* 2000)

1.4 IFN-γ production and cell stimulation with antigens

In vivo infection with *M. bovis* leads to antigen presentation on the cell membrane of antigen presenting cells (APCs). An antigen specific T-cell can detect these antigens and in reaction will start to produce IFN-γ (Figure 1.2). When isolated peripheral blood mononuclear cells (PBMC's) or whole blood from the animal is stimulated with certain antigens specific for *M. bovis,* memory T-cells in the PBMC's or the whole blood will produce IFN-γ specific to those antigens (Figure 1.2). If however the blood is extracted from an animal that was not exposed to *M. bovis*, IFN-γ specific to those antigens will not be produced. When mitogens, such as poke weed mitogen (PWM) or phytohaemagglutinin (PHA), are added to PBMCs or whole blood they induce the T-cells to produce many cytokines, one of which is IFN-γ.

1.5 The IFN-γ capture ELISA

An IFN-γ capture ELISA (Figure 1.3) detects the presence of IFN-γ from samples that were stimulated with mitogens or specific antigens. The wells of an ELISA plate are coated with the capture antibody specific for the antigen that has to be tested. In this case it is specific for IFN-γ. In the next step the supernatant from the sample, stimulated with an antigen or a mitogen, is added to the wells. If the animal has been exposed to *M. bovis*, after stimulation with PPDB the supernatant should contain IFN-γ. Detection of the IFN-γ is determined with the detecting antibody. The addition of a conjugate and finally the substrate will yield a colour reaction in the presence of IFN-γ. The intensity of reaction is determined by reading the optical density ($OD_{490\ nm}$).

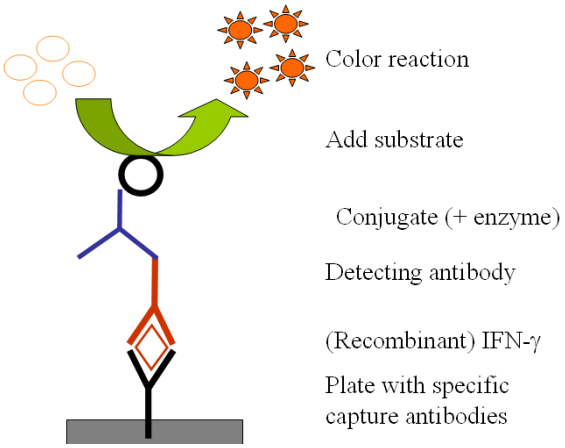

Figure 1.3 Diagrammatic representation of a capture ELISA (Maas 2008)

1.6 Aims of this study

This study had two aims.

1. To determine if a former ELISA developed for the detection of rhinoceros IFN-γ (Morar *et al.* 2007) could be used for the detection of recombinant and native feline IFN-γ.
2. The IFN-γ ELISA that was adapted (Rhodes *et al.* 2008) for use in felines will be performed to determine repeatability and will be used to test cat and lion samples for the detection of native IFN-γ.

2. Materials and Methods

2.1 Sample collection

2.2.1 Onderstepoort

Cat blood samples (n = 3) were provided by the Onderstepoort Veterinary Academic Hospital (OVAH). The blood was processed for the isolation of PBMCs and for whole blood simulations.

2.2.2 Kruger National Park

Blood was collected from a total of 47 lions in both EDTA and heparin tubes (Figure 2.1). The blood from the EDTA tubes was processed for PBMC isolation. The blood from the heparin tubes was used for whole blood stimulations. All samples were processed at the laboratories at the Wildlife Veterinary Services (WVS) in Skukuza.

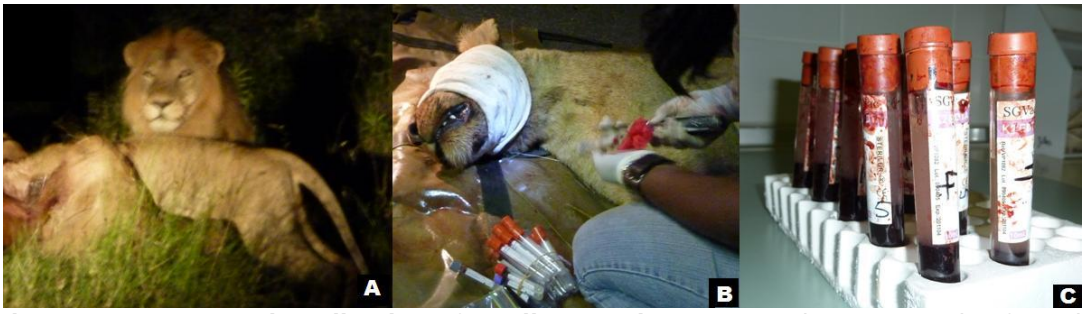

Figure 2.1 Sample collections from lions at the KNP. A. Lionesses eating from the bait while the male looks up. **B**. Blood is drawn from a sedated lioness. **C**. Blood samples collected from one of the captures.

2.2 Whole blood stimulation

Prior to whole blood culturing each heparin tube was mixed by inverting the tubes several times, and 1 ml aliquots were dispensed into the wells of a 24-well tissue culture plate (Figure 2.2). This was followed by the addition of mitogens and protein derivates (Table 2). Wells containing only blood or only medium without the addition of mitogens or PPDs were included as negative controls. As additional negative controls, the same mitogen concentrations were added to medium (RPMI 1640) and treated as the test samples. This control would serve in indicating, in the ELISA, if a signal obtained after performing the ELISA was indeed induced by the stimulated

cells and not by the mitogens themselves. The wells were mixed by gently swirling the culture plates and were incubated at 37^{o}C. For various time periods the supernatant and the medium controls for each condition were harvested (Table 2). The supernatants were stored at -20^{o}C until further use.

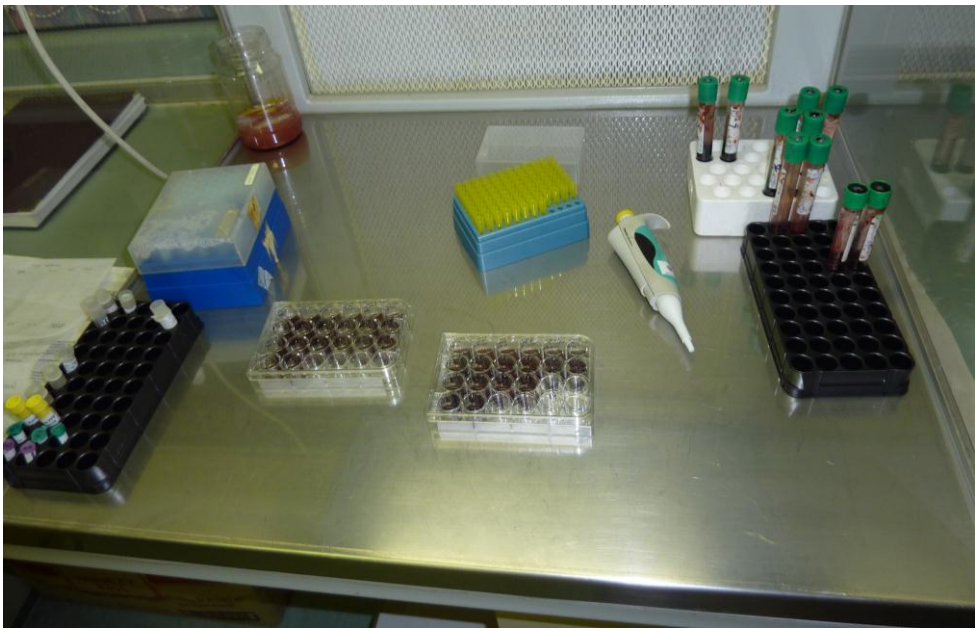

Figure 2.2 Blood was aliquoted into 24 well culture plates

The cat samples were stimulated with 10 µg/ml ConA, 10 µg/ml PWM and a negative control to which no mitogen was added. The supernatant was harvested after 72, 96 and 120 hr.

Table 2: Whole blood and medium test conditions.

Mitogens/Antigens	Mitogen/Antigen concentration	Incubation time (hr)
1. PWM	10 µg/ml	24, 48, 72, 96, 120
2a. SEB	10 µg/ml	24, 48, 72, 96
2b. SEB	25 µg/ml	24, 72
3a. ConA	10 µg/ml	24, 48, 72, 96
3b. ConA	25 µg/ml	24, 72
4. PHA	10 µg/ml	24, 48, 72, 96
5a. ESAT-6/CFP-10	5 µg/ml	24, 48, 72, 96
5b. ESAT-6/CFP-10	10 µg/ml	24, 48, 96
6a. PPD A	10 µg/ml	24, 48, 96, 120
6b. PPD A	15 µg/ml	48, 72
6c. PPD A	30 µg/ml	24, 48, 72, 96
7a. PPD B	10 µg/ml	24, 48, 96, 120
7b. PPD B	15 µg/ml	48, 72
7c. PPD B	30 µg/ml	24, 48, 72, 96
8a. PPD F	10 µg/ml	24, 48, 96
8a. PPD F	15 µg/ml	48, 72
8c. PPD F	30 µg/ml	24, 48, 72, 96

PWM: poke weed mitogen, SEB: staphylococcal enterotoxin B, ConA: Concanavalin A , PHA: phytohaemagglutinin, ESAT-6/CFP-10: early secretory proteins of *Mycobacterium tuberculosis*, PPD A: avian purified protein derivate , PPD B: bovine purified protein derivate , PPD F: fortuitum purified protein derivate.

2.3 PBMC isolation

The blood collected in EDTA vacutainer tubes was centrifuged at 2000 rpm for 10 min. without a brake, to collect the buffy coat. The buffy coat was then pipetted into a clean tube and kept on ice. Phosphate buffered saline (PBS) solution was added to obtain equal volumes (10 ml) of the buffy coat PBS mix. The same volume of Histopaque was added to a clean tube and the buffy coat/PBS solution was gently layered on to the surface of the Histopaque. These tubes were centrifuged for 25 min. at 1200 rpm (no brake). The cloudy layer of cells were collected and resuspended in 5 ml of PBS, followed by centrifugation step for 10 min. at 1000 rpm (with brake). The wash step was repeated. The supernatant was discarded and the pellet was re-suspended in 1 ml culture medium (RPMI + L-glutamine + 1% Pen/Strep) (Figure 2.3). To determine the number of cells (cells/ml) isolated, they were stained with Trypan blue solution and counted using a hemocytometer.

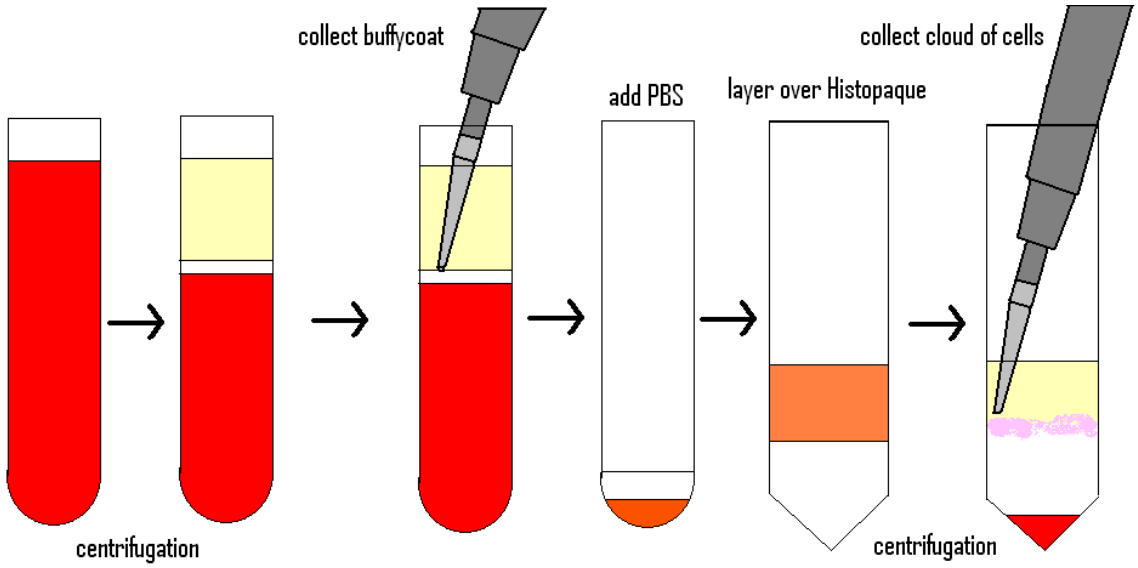

Figure 2.3 Diagrammatic representation of the isolation of PBMCs

2.4 PBMC stimulation

Depending on the amount of PBMCs isolated they were either diluted or concentrated to obtain a final concentration of 1 X 10^6 cells/ml. These cells were then aliquoted to wells of a 24-well tissue culture plate and antigens, mitogens or PPDs were added to the respective wells. The plate was gently swirled to mix the contents and placed in a 37^{o}C incubator until harvesting. As in the whole blood samples (see section 2.2) medium controls containing different concentrations of mitogens were also included. The supernatant from the stimulated PBMC's and their medium controls were harvested after 24, 48, 72 and 96 hr of incubation (Table 3), and were stored in cryotubes at -20°C until further use.

Table 3: PBMC and medium test conditions

Antigens	Antigen concentration	Incubation time (hr)
1a. PWM	5 µg/ml	24, 48
1b. PWM	10 µg/ml	24, 48, 72, 96
2a. SEB	10 µg/ml	24, 48, 72, 96
3a. ConA	10 µg/ml	24, 48, 72, 96
5a. ESAT-6/CFP-10	5 µg/ml	24, 48
6a. PPD A	10 µg/ml	24, 48, 72, 96
7a. PPD B	20 µg/ml	24, 48, 72, 96
7b. PPD B	10 µg/ml	24, 48
8a. PPD F	20 µg/ml	24, 48, 72, 96

The cat samples were treated with 10 µg/ml PWM, 10 µg/ml Concanavalin A (Con A) and an unstimulated control. The supernatant was harvested after 72, 96 and 120 hr.

2.5 Recombinant IFN-γ ELISAs

A polysorb ELISA plate (Nunc) was coated with 0.25 µg capture antibody (ID11) per well. After an overnight incubation at 4ºC, the buffer was discarded and the wells were blocked with 2% milk powder in PBS (100 µl/well) and incubated at 37°C for one hr. The block buffer was discarded and the wells were washed five times with distilled water using an ELISA plate washer. Several dilutions (2000, 1000, 500, 250, 62, 31, 16, 8, 4, 2, 1 and 0 ng/well) of recombinant rhinoceros, equine and feline IFN-γ were added to the wells in volumes of 50 µl. After one hr of incubation at 37°C the fluids were discarded and the plate was washed five times with distilled water containing 0.1% Tween 20 using an ELISA plate washer. Detecting antibody (IgY, 1 µg/ml) was then added to each well and incubated at 37°C for one hr. After repeating the washing step HRP labeled conjugate (rabbit anti-chicken, 1:3000) was added to each well. The plate was incubated at 37°C for one hr. The ó-phenylenediamine dihydrochloride (OPD) substrate was prepared by dissolving 1 tablet (5 mg) of OPD in 5 ml citrate buffer and 2.5 µl H_2O_2. After another wash step (5 x distilled water + 0.1%Tween 20), 50 µl OPD-substrate was added to each well and incubated at room temperature for 30 min. After the stop solution (2M H_2SO_4, 50 µl/well) was added the plate was read using an ELISA-reader at an OD of 492 nm.

Following the protocol as mentioned above, a second ELISA was performed to determine the optimal concentrations of capture and detecting antibody. Three different concentrations of ID11 (0.5, 0.25 and 0.05 µg/well) and two different concentrations of IgY (0.25 and 0.05 µg/well) were used. A dilution series, starting with 250 ng/well of the recombinant feline IFN-γ was performed.

A third ELISA, following the same protocol as mentioned above, was performed to determine detection of native cat and lion IFN-γ. The supernatant from the processed samples and from the positive and negative controls were added to different wells. Recombinant feline and rhinoceros IFN-γ were used as the positive controls. Negative controls included wells without recombinant IFN-γ, medium with mitogen and serum samples.

2.6 Adapted feline IFN-γ ELISAs (Rhodes *et al.* 2008)

The wells of a Maxisorp ELISA (Nunc) plate was coated with 1 μg/ml of the capture antibody (AF764, R&D System Europe Ltd., U.K.) diluted in carbonate buffer (pH 9.6). The coating was completed by an overnight incubation at 4°C. The plate was blocked with block buffer one (BB1 -4% BSA in PBS) for one hr at 37°C and then washed (3 times) with PBS containing 0.05% Tween 20. Recombinant feline IFN-γ (764-FG/CF, R&D Systems) diluted in BB1 and then added to the respective wells in a two times dilution series. The dilution series began with 20 µg/ml. After one hr incubation at 37°C and a wash step, 50 µl of a solution of 1 µg/ml detecting antibody (BAF764 in block buffer, R&D Systems) was added to each well. After further incubation at 37 °C for 1 h the plate was washed. The conjugate, streptavidin-alkaline phosphatase (0369, DAKO, U.K.), was diluted 1:1000 and 1:2000 in block buffer. Two dilutions were used to determine the optimal concentration of the enzyme. After addition to respective wells the plate was incubated for one hr at 37°C. The plate was washed and 50 µl of 1-step^{TM}PNPP substrate was added to each well. The substrate was prepared with 4 ml distilled H_2O, 1 ml DEA buffer and 1 tablet phosphatase substrate. An incubation of one hr at room temperature took place after which a stop solution (2 M EDTA) was added and the plate was read using an ELISA-reader at 405 nm.

The second feline IFN-γ ELISA was performed in the same way, except that only the 1:2000 dilution of conjugate was used. Whole blood and PBMC cat samples stimulated with PWM, Con A and an unstimulated control were tested. All samples were incubated for 96 hr. As negative controls, medium (RPMI + glutamine) incubated with mitogens, serum and block buffer were also used.

3. Results

3.1 Recombinant IFN-γ ELISAs

The results from the recombinant IFN- γ ELISA are shown (Figure 3.1). The results indicate that all recombinant antigens used in this ELISA are detected. The yellow line indictes the OD values of the standard curve for the recombinant feline IFN-γ (rFelIFN-γ).

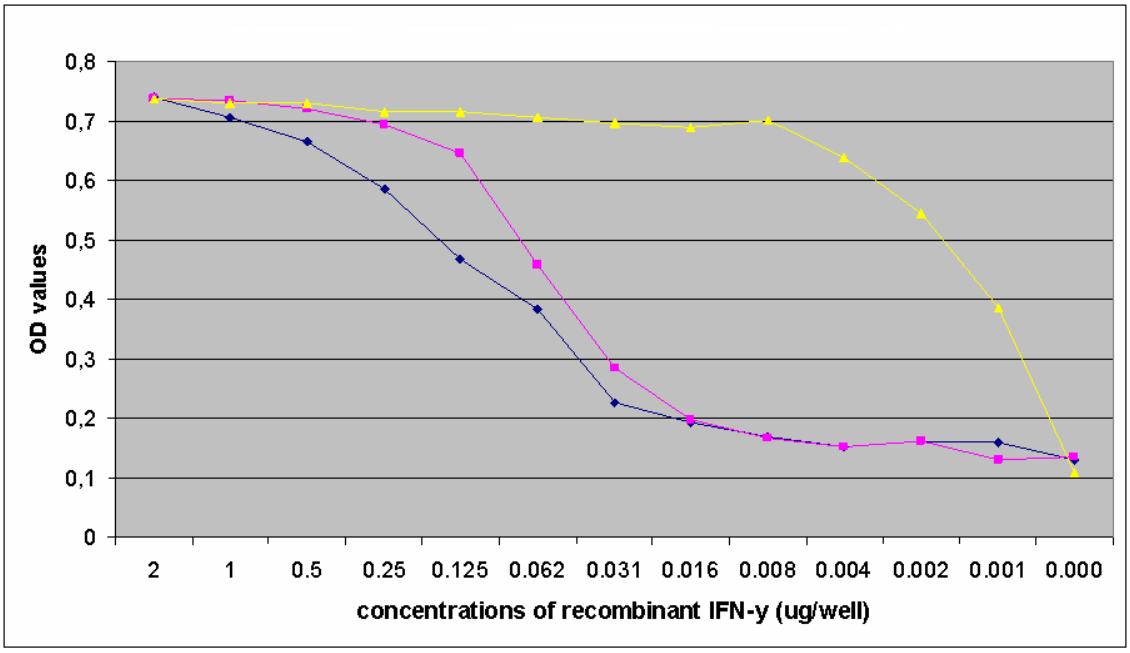

Figure 3.1 Recombinant equine, rhino and feline IFN-γ ELISA

In Figure 3.2 the OD values of eight different concentrations of recombinant feline IFN-γ are depicted in combination with two different concentrations of IgY and three concentrations of 1D11. These results indicate that the optimal concentration of capture (1D11) antibody is 5/10 µg/ml when using 1 µg/ml of detecting antibody (IgY) respectively. Since there was no significant difference when using 5 or 10 µg/ml of the capture antibody, it was decided to continue using 5 µg/ml of the capture antibody in further ELISAs.

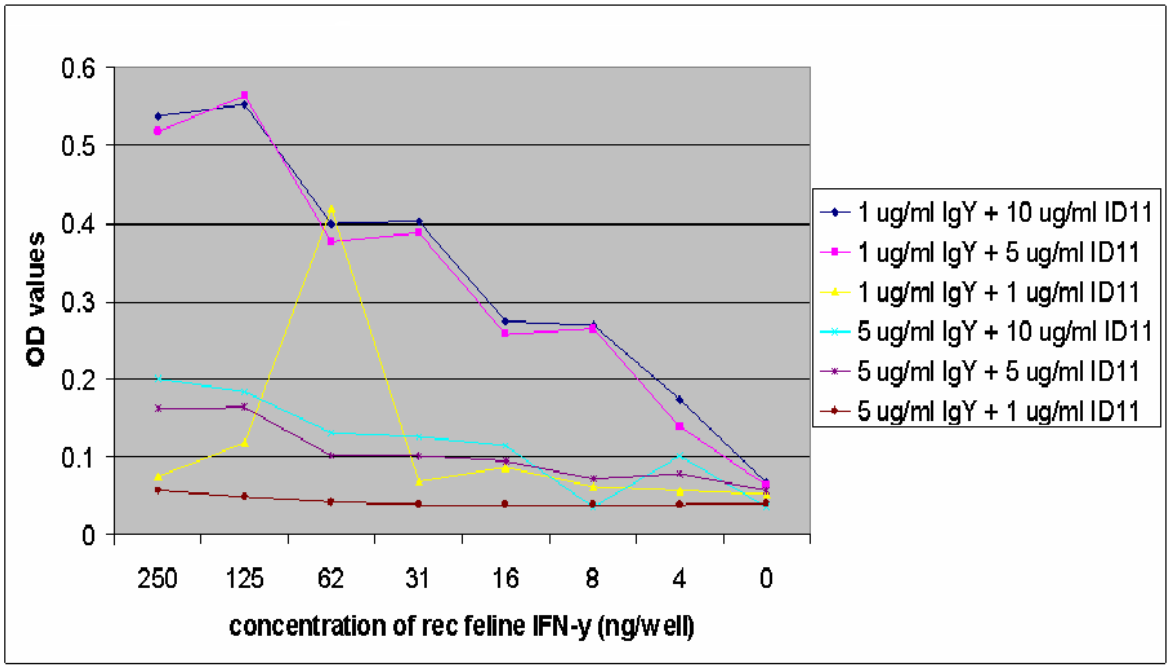

Figure 3.2 Titration ELISA's for detection of rFelIFN-γ

3.2 Adapted Feline IFN-γ ELISA

Both dilutions of conjugate (1:1000 and 1:2000) gave high OD values at a concentration of 1000 ng/well feline IFN-γ, respectively 3.5 and 3. They decrease slightly till a concentration of 62 ng/well feline IFN-γ and then start to drop faster reaching an OD value around 1 with a concentration of 2 ng/well feline IFN-γ (Figure 3.3). The line that indicates the 1:1000 dilution of conjugate (pink) has higher values than the line from the 1:2000 dilution of conjugate (blue). In both lines the OD values at 1000 ng/well IFN-γ and 0 ng/well IFN-γ differ enough from each other to be able to say that with both dilutions IFN-γ can be detected. This means that with both dilutions of conjugate (1:1000 and 1:2000) IFN-y can be detected.

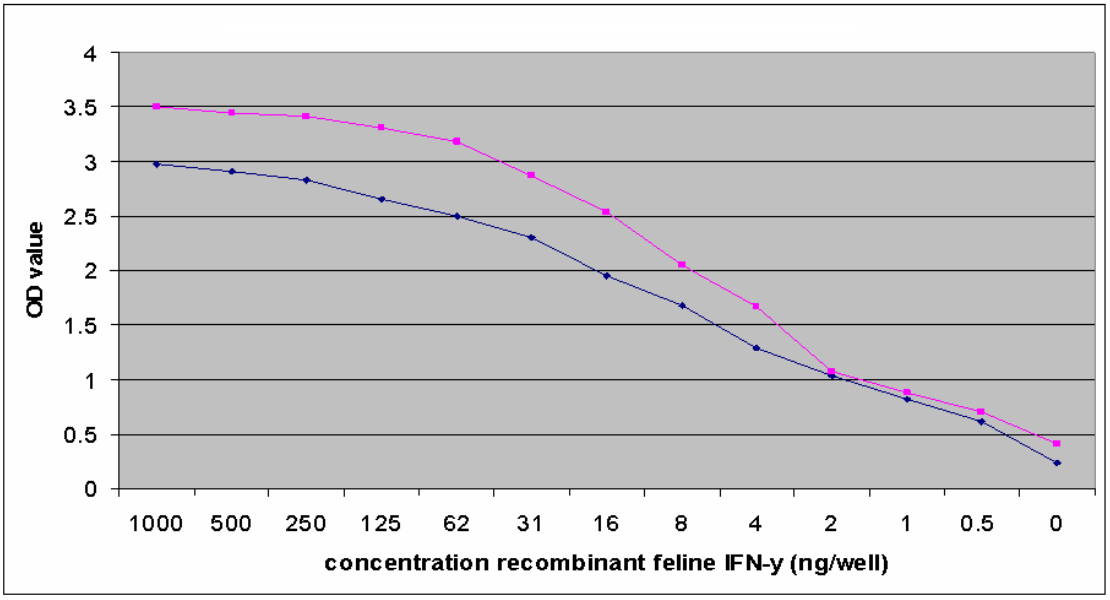

Figure 3.3 Adapted feline IFN-y ELISA using two different conjugate dilutions

4. Discussion

4.1 PBMC Isolation

Prior to using the protocol described in this report a previously adapted protocol for the isolation of PBMCs was used but proved difficult when used on lion blood samples that were collected in the Kruger national park (see section 2.2.2). The cloudy layer above the Histopaque, which usually consists of the PBMC layer, did appear but did not seem to contain only cells. It was thought that this layer consisted of proteins, lipids and contained a fibrin network. The higher level of proteins and lipids in the lion blood could be due to the fact that most of the lions had consumed some of the bait during the capture. The reason for the fibrin network could be that the centrifugation steps rendered the thrombocytes unstable and activated them resulting in blood clots. As a result of these two factors no clear separation of PBMC's appeared and the cells could not be extracted.

The protocol for the PBMC isolation was therefore amended to prevent this from happening, and the centrifugation speed was decreased from 2800 to 1200 rpm for the Histopaque step. To prevent cell degradation the centrifugation speeds, used for the wash steps, were also reduced from 1200 to 1000 rpm. These changes greatly improved the isolation of PBMCs from the lion blood samples.

4.2 IFN-γ ELISAs

Two different IFN-γ ELISA's were used in this study to determine first time conditions towards developing an assay for the detection of *M. bovis* infection in lions.

The first aim was to use an already developed IFN-γ ELISA for rhinoceroses (Morar *et al.* 2007) to determine detection of recombinant feline IFN-γ. If this proved successful the next step would be to determine detection of native cat and lion IFN-γ. Although detection of recombinant feline IFN-γ proved to be successful, detection of IFN-γ from mitogen stimulated blood and PBMC samples were unsuccessful and no conclusive results were obtained. With further studies, if this proves successful, this ELISA could be optimised for the use in domestic and wild felid species. The first step in this process would be to use the optimised conditions on domestic cat samples and then move on to the lion samples that were collected and processed in this study.

The second IFN-γ ELISA used in this study was the adapted feline IFN-γ ELISA (Rhodes *et al.* 2008), however due to time constraints and limited recourses this ELISA did not provide the study with conclusive results. This ELISA has the potential of being used as the current test in diagnosing BTB in wild felids (lions), if it proves to detect both recombinant and native lion IFN-γ. The drawback of this is that the reagents are supplied in small quantities and can prove to be rather expensive, especially in the initial trials of optimizing the ELISA, before field samples can be tested.

As a future objective, a comparison of the two ELISA's will be performed to determine which of the two ELISAs is most suitable for optimising an ELISA for the detection of *M. bovis* infections in lions.

A lion specific IFN-γ assay would be the next step at developing a diagnostic assay for use in these cats. The gene of interest was sequenced by Maas *et al*. (2010) and can be cloned and expressed to produce recombinant lion IFN-γ. This is a basis to develop a specific IFN-γ ELISA for use in lions.

5. Conclusions

The results from this study indicate that the rhinoceros IFN-γ ELISA can detect feline recombinant IFN-γ. Although these experiments have to be repeated, the initial results indicate that the detection limit is about 125 ng of recombinant protein/50 μl.

Results for the adapted feline IFN-γ ELISA in this study indicate that this ELISA can be reproduced under the laboratory conditions set at the Department of Veterinary Tropical Diseases (DVTD) of the University of Pretoria.

However, further work has to be done to determine more detailed conditions on optimizing the ELISA for use in lions. Once both ELISAs are optimized a comparison can be made between the two ELISAs.

6. Acknowledgements

First of all I want to thank Prof. Dr. V. Rutten for the opportunity to participate in this research project in South Africa, and for his efforts and advice. To Dr. Morar, thank you for your efforts, advice and friendly collaboration. To Prof. Dr. A. Michel, who provided me with useful remarks towards my research and presentation, thank you.

I would like to thank the University of Utrecht and the University of Pretoria for the financial support required to undertake this research project.

My appreciation also extends to the Bureau Internationale Contacten (BIC) for the guidance in assisting me in identifying possible research projects. In conjunction with the administrative staff at the DVTD, BIC also assisted me in finding suitable accommodation in Pretoria.

I would like to thank the Department of Veterinary Tropical Diseases for the use of their facilities and the co-operation of the laboratory personnel.

For the collection of the blood from domestic cats I would like to thank the Onderstepoort Veterinary Animal Hospital (OVAH).

I would also like to thank the veterinarians and staff at the Wildlife Veterinary Services (VWS) at Skukuza for the amazing experience, the use of their labs and for the collection of the lions blood samples.

A special note of appreciation to veterinarians Dr. D. Govender, for her hospitability and co-operation in the lab, and to Dr. P. Buss for being given the opportunity to participate in the captures.

7. References

Andersen, P., Munk, M, Pollock, J.and Doherty, T., 2000. Specific immune-based diagnosis of tuberculosis. *The Lancet,* **356**, 1099-1104.

Bengis, R. G., Kriek, N. P. J., Keet, D. F., Raath, J. P., de Vos, V., Huchzermeyer, H., 1996. An outbreak of tuberculosis in a free living African buffalo (*Syncerus caffer SPARRMAN*) population in the Kruger National Park: a preliminary report. *Onderstepoort Journal of Veterinary Research,* **63**, 15 -18.

Demissie, A., Leyten, E.M., Abebe, M., Wassie, L., Aseffa, A., Abate, G., Fletcher, H., Owiafe, P., Hill, P. C., Brookes, R., Rook, G., Zumla, A., Arend, S. M., Klein, M., Ottenhoff, T. H., Andersen, P., Doherty, T. M. & the VACSEL Study Group, 2006. Recognition of stage-specific mycobacterial antigens differentiates between acute and latent infections with *Mycobacterium tuberculosis*, *Clinical and Vaccine Immunology : CVI,* **13**, 179-186.

Engeleberg, N. C., DiRita, V., Dermody, T. S. 2007. Chapter 23, *Mycobacteria*: *Tuberculosis* and *Leprosy* by David Haas. Schaechter's Mechanisms of Microbial Disease. P.p. 251 -260.

Gupta, S., Bandyopadhyay, D., Paine, S. K., Guta, S., Banerjee, S., Bhattacharya, S., Gachhui, R., Bhattacharya, B., 2010. Rapid Identification of *Mycobacterium* Species with the Aid of Multiplex Polymerase Chain Reaction (PCR) From Clinical Isolates. *The Open Microbiology Journal,* **4***,* 93-97.

Keet, D. F., Kriek, N. P., Penrith, M. L., Michel, A. and Huchzermeyer, H. 1996. Tuberculosis in buffaloes (*Syncerus caffer*) in the Kruger National Park: spread of the disease to other species. *The Onderstepoort Journal of Veterinary Research,* **63**, 239-244.

Keet, D. F., Michel, A. and Meltzer, D. G. A., 2000. Tuberculosis in free-ranging lions (*Panthera leo*) in the Kruger National Park. *Proceedings of the South African Veterinary Association Biennial Congress, Durban, Kwazulu-Natal,* 232-241.

Keet, D. F., Michel, A. L., Bengis, R. G., Becker, P., van Dyk, D. S., van Vuuren, M., Rutten, V. P. M. G. and Penzhorn, B. L., 2010. Intradermal tuberculin testing of wild African lions (*Panthera leo*) naturally exposed to infection with *Mycobacterium bovis*. *Veterinary Microbiology*, **144**, 384–391.

Maas, M., 2008. Tuberculosis in lions (*Panthera leo*) in South Africa: Evaluation of the immune response towards *Mycobacterium bovis*. *Excellent Track Research.*

Maas, M., Rhijn, I., Allsopp, M.T.E.P. and Rutten, V.P.M.G., 2010. Lion (*Panthera leo*) and cheetah (*Acinonyx jubatus*) IFN-γ sequences*. Veterinary Immunology and Immunopathology,* **134**, 296-298.

Michel, A. L., 2002. Implications of tuberculosis in African wildlife and livestock. *Annals of the New York Academy of Sciences,* **969**, 251-255.

Michel, A. L., Bengis, R. G., Keet, D. F., Hofmeyr, M., Klerk, L. M., Cross, P. C., Jolles, A. E., Cooper, D., Whyte, I. J., Buss, P., Godfroid, J., 2006. Wildlife tuberculosis in South African conservation areas: implications and challenges. *Veterinary Microbiology*, **112**, 91–100.

Michel, A. L., 2008. *Mycobacterium Fortuitum* Infection Interference with *Mycobacterium bovis* Diagnostics: Natural Infection. *Journal of Veterinary Diagnostic Investigation*, **20**, 501-503.

Michel, A. L., Cooper, D., Jooste, J., de Klerk, L.-M., Jolles, A., 2008. Approaches towards optimizing the gamma interferon assay for diagnosing *Mycobacterium bovis* infection in African buffalo *(Syncerus caffer*). *Preventive Veterinary Medicine*, **98**, 142-151.

Michel, A. L., Coetzee, M. L., Keet, D. F., Mare´, L., Warren, R., Cooper, D., Bengis, R. G., Kremer, K., van Helden, P., 2009. Molecular epidemiology of *Mycobacterium bovis* isolates from freeranging wildlife in South African game reserves. *Veterinary Microbiology*, **133,** 335–343

Morar, D., 2009. Development of ELISAs for the detection of interferon-gamma in rhinoceroses and elephants as a diagnostic tool for *Mycobacterium bovis* and *Mycobacterium tuberculosis* infections, PhD *thesis.*

Noordhoek, G. T. , Kolk, A. H. , Bjune, G. , Catty, D. , Dale, J. W. , Fine, P. E. , Godfrey-Faussett, P. , Cho, S. N. , Shinnick, T. , Svenson, S. B. , Wilson, S. and Embden, J. D. A. V., 1994. Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among seven laboratories. *Journal of Clinical Microbiology,* **32**, 277-284.

Quin, P.J., Markey, B.K., Carter, M.E., Donnelly, W.J. and Leonard, F.C., 2002. Chapter 17, *Mycobacterium* species.*Veterinary Microbiology and Microbial Disease*. p.p. 97-105.

Rhodes, S. G., Gruffydd-Jones, T., Gunn-Moore, D. and Jahans, K., 2008. Adaptation of IFN-gamma ELISA and ELISPOT tests for feline tuberculosis. *Veterinary Immunology and Immunopathology,* **124***,* 379-384.

de la Rua-Domenech, R., Goodchild, A. T., Vordermeier, H. M., Hewinson, R. G., Christiansen, K. H. and Clifton-Hadley, R. S., 2006. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. *Research in Veterinary Science,* **81**, 190-210.

Sankar, S., Ramamurthy, M., Nandagopal, B., Sridharan, G., 2011. An appraisal of PCR-based technology in the detection of *Mycobacterium tuberculosis*. *Molecular Diagnosis and Therapy*. **15**, 1-11.

de Vos, V., Raath, J. P., Bengis, R. G., Kriek, N. J. P., Huchzermeyer, H., Keet, D. F., Michel, A., 2001. The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa. *Onderstepoort Journal of Veterinary Research,* **68**, 119 -130.

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