Optimization of the Rhinoceros interferon-gamma ELISA for the diagnosis of bovine tuberculosis in Rhinoceroses

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1. Abstract

Mycobacterium bovis, the causative agent of bovine tuberculosis (BTB), is known to infect many different species and is a big problem in South Africa's wildlife. Black and white rhinoceroses are critically endangered and near threatened species respectively, and are known to be susceptible to *M. bovis*. The aim of this study was to optimize the IFN-γ assay that was developed for the diagnosis of BTB in rhinoceroses.

Since Rhinoceros IFN-γ and horse IFN-γ were highly homologous and both recognised in the assay, horse blood was used for the optimization experiments. The optimal concentration of capture and detection antibody for the capture ELISA was determined as well as the optimal concentration of recombinant rhinoceros IFN-γ and horse IFN-γ that serves as a positive control for the assay. Whole blood (WB) and peripheral blood mononuclear cells (PBMCs) samples were stimulated with different mitogens in varying concentrations to test production of IFN-γ. Results showed no difference in signal between WB and PBMC samples. Pokeweed mitogen (PWM) and Phytohemagglutinin (PHA) gave the strongest signals in all concentrations tested. Different incubation times were tested to see if longer incubation of the samples would enhance IFN-γ production. This revealed that 24 hours is the optimal incubation time before the samples can be tested in the ELISA and that longer incubation does not improve the strength of the signal. Further testing of the IFN-γ ELISA optimized in this study with rhinoceros blood is necessary. This ELISA, can in addition be used as a possible diagnostic tool for the detection of horse IFN-γ for other diseases.

2. Introduction

2.1 *Mycobacterium bovis*

Bovine tuberculosis (BTB), caused by *Mycobacterium bovis*, is commonly known as a disease of livestock. In the year 1990 (Bengis *et al.* 1996), the pathogen was identified in African buffaloes (*Syncerus caffer*) in the Kruger National Park (KNP) in South Africa (Michel 2008). It is most likely that *M. bovis* was transmitted from infected cattle to buffaloes by aerosols. African buffaloes are now recognised as an important wildlife maintenance host of *M. bovis*. Bovine TB has been diagnosed in different wildlife species in South Africa, including lions (*Panthera leo)*, cheetahs *(Acinonyx jubatus)*, kudus *(Tragelaphus strepsiceros)*, impalas *(Aepyceros melampus)* and leopards *(Panthera pardus)* (Michel 2006). South Africa's wildlife is of great economical and social importance, and once infected can serve as a reservoir for transmission of *M. bovis* to domestic species and humans. Therefore, it is of great importance that strategies be devised to identify infection in animals and thus control the spread of the infection to other animals (Michel 2008).

2.2 BTB in Rhinoceroses

Black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses are a critically endangered and a near threatened species (*www.iucnredlist.org*) respectively. Cases of *M. bovis* infections have been documented in captive black and white rhinoceroses, showing that these species are susceptible to tuberculosis (Dalovisio *et al.* 1992, Stetter *et al.* 1995, Mann *et al.* 1981). Espie *et al.* (2009) recently reported a case of *M. bovis* infection in a free-ranging black rhinoceros from the NZG's Mokopane Biodiversity Conservation Centre in Mokopane, Limpopo Province. *M. bovis* was confirmed to be the causative agent by culture and PCR. Little is known about the exact cause of the disease in this animal. Clinical signs in *M. bovis* infected rhinoceroses were reported to be (intermittent) diarrhoea, severe weight loss, stranguria, coughing and clear nasal discharge. The most important findings at necropsy were severe granulomatous lesions in the lungs (granulomatous pneumonia) (Daloviso *et al.*1992, Espie *et al.* 2006).

2.3 Immune response

The primary response to mycobacteria is known to be a cell-mediated immune response (CMI) (Pollock *et al.* 2001). The main host cells for mycobacteria are macrophages. The bacteria either remain hidden in the macrophages where they are able to resist the immune system, or are eradicated due to immune reactivity i.e. in the lymph nodes. Macrophages activate T-helper-1 (Th1) cells, which in turn produce and release interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). The cytokine IFN-γ, mainly produced by CD4 T cells, plays an important role in the early detection of an *M. bovis* and *M. tuberculosis* infection (Flynn *et al.* 1993, Anderson *et al.* 2007).

IFN-γ activates macrophages, thus containing the infection, and increases the expression of MHC class I and II molecules. In this way, it enhances antigen presentation. Infection with mycobacteria may become latent if the infection is contained within the host. Latent infected animals can become clinically ill in course of time, most likely due to a shift to the humoral immune response. Antibodies are not able to destroy the mycobacteria inside macrophages. This leads to multiplication of mycobacteria, which causes clinical disease (Pollock and Neill 2002).

2.4 Diagnostic tests

2.4.1 The intradermal skin test

The intradermal skin test (IDT) is used for the diagnosis of BTB in cattle. The skin test is based on the delayed hypersensitivity reaction upon injection of tuberculins. There are two forms of this test, the single intradermal skin test (SIT) and the single intradermal comparative tuberculin test (SICTT). In the SIT, the tuberculin bovine protein purified derivative (PPD) is used. In the SICTT, avian PPD is used in addition to bovine PPD. In this way, a differentiation can be made between animals that are infected and animals that have been in contact with environmental mycobacteria. When a swelling is seen 72 hours after injection of the tuberculins, this indicates that the animal has been exposed to mycobacteria. If the swelling to bovine PPD is more than 4 mm greater than the swelling to avian PPD, the animal is considered to be infected with BTB (OIE 2004). The

disadvantages of using the IDT on wildlife include recapture of animals, which causes stress to the animals, it is expensive and these tests have not been validated in rhinoceroses.

2.4.2 Serology based tests

Serology-based tests depend on the presence of certain antibodies in the animal after cell-mediated immunity (CMI) no longer plays a role. A rapid test, recently developed by Lyashchenko *et al*. (2008), allows for the quick detection of antibodies against several antigens. As stated earlier, the humoral immune response plays a role in the later stage of an *M. bovis* or *M. tuberculosis* infection and thus such a test is valuable when detecting infected animals or those animals already shedding the pathogen.

2.4.3 IFN-γ ELISAs

An IFN-γ-based test, Bovigam™, is widely used for diagnosis of BTB in cattle. The IFN-γ assay has proven to be very successful in the early detection of *M. bovis* infections in cattle (Wood & Jones 2001, Gormley *et al.* 2006). This assay consists of two phases, first the IFN-γ production by T-cells is stimulated using tuberculins, and next the IFN-γ produced is measured in an IFN-γ specific capture ELISA (Wood & Jones 2001). For wildlife, the IFN-γ assay would be an ideal test, since animals only have to be captured once. However, the test only recognizes bovine IFN-γ and reagents need to be produced for different wildlife species. Interferon-γ assays have already been developed for, amongst others, elephants and rhinoceroses (Morar 2009). Dalley *et al.* (2008) developed an IFN-γ assay for the European badger *(Meles meles)*, an important wildlife host of *M. bovis* in Britain.

2.4.4 Bovine TB diagnostics in rhinoceroses

It is of great importance that a diagnostic tool becomes available for rhinoceroses. It is important to diagnose the infection as early as possible, before the animals show clinical signs, so *M. bovis* or *M. tuberculosis* infected rhinoceroses can be quarantined and treated before the disease can be spread to other animals. To date, there is no validated test available for the detection of tuberculosis in pachyderms, such as rhinoceroses. The IFN-

γ ELISA for the diagnosis of BTB in rhinoceroses, developed by Morar *et al.* (2007), and the use of a rapid antibody test for the diagnosis of *M. bovis* in elephants, reported by Lyashchenko *et al.* (2006), are promising. However, further research is necessary to develop sensitive and specific diagnostics for these animals.

2.5 Aim and objectives

The aim of this study was to optimize the recombinant Rhinoceros IFN-γ ELISA, developed for the diagnosis of *M. bovis* and *M. tuberculosis* infections in rhinoceroses (Morar *et al.* 2007). Since rhinoceros blood could not be obtained during this study horse blood was used for the optimization experiments. Previous results indicated that the rhinoceros and horse IFN-γ amino acid sequences share a 90% identity (Morar *et al.* 2007) and are both recognised in the rhinoceros IFN-γ ELISA.

The following conditions were evaluated:

- Optimal concentrations of capture and detecting antibody
- Detection limit of recombinant horse and rhinoceros IFN-γ that can be detected by the ELISA (detection limit of the ELISA)
- The most suitable mitogen and its concentrations for the stimulation of both whole blood (WB) and peripheral blood mononuclear cells (PBMCs), which will then be used as a positive control in the ELISA
- Optimizing the ELISA for the detection of IFN-γ from mitogen stimulated WB and PBMC

3. Materials and Methods

3.1 PBMC isolation and stimulation

Blood from horses were collected in heparin tubes. To obtain the buffy coat the tubes were centrifuged at 2000 rpm/20°C for 10 min. The buffy coat was then pipetted into clean 50 ml tubes. The volumes were adjusted to 10 ml with medium (RPMI 1640 + L-glutamine). Histopaque (10 ml) was added to a 50 ml tube (Blood : Histopaque = 1 : 1) and the blood/medium mix was gently layered on top of the Histopaque. The tubes containing the blood/Histopaque gradient were centrifuged for 25 min at 1200 rpm (20°C). The PBMC interphase was pipetted into a clean tube and placed on ice. Phosphate buffered saline solution (PBS) was then added to the tubes to adjust the volumes to 3 ml. The cells were then washed by centrifugation for 10 min at 1000 rpm (4°C). The PBS was discarded and the wash step was repeated. After discarding the PBS, the cell pellet was carefully resuspended with culture medium (RPMI & 5% Penicillin/Streptomycin (Pen/Strep) & 10% foetal calf serum (FCS)). To determine the number of cells isolated, a cell count was performed using a hemacytometer. Depending on the amount of cells counted, the samples were diluted in culture medium and 1 X 10^6 cells/ml were aliquotted to wells of a 24-well tissue culture plate and stimulated with varying concentrations (0 µg/ml, 0.5 µg/ml, 1 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml, 25 µg/ml & 50 µg/ml) of different mitogens (Concanavalin A (ConA), *Staphylococal* enterotoxin B (SEB), Pokeweed mitogen (PWM) and Phytohemagglutinin **(**PHA)). The plates were incubated at 37°C with 5 % CO_2. The supernatants were harvested at 24, 48 and 72 hrs and used either in an ELISA or stored at -20°C until further use.

3.2 Whole blood stimulation

Horse blood was collected in heparin BD Vacutainer tubes and 1 ml was pipetted into individual wells of a 24-well tissue culture plate and stimulated with different mitogens in varying concentrations as described in 3.1. The plates were incubated for 24, 48 and 72 hrs at 37°C with 5 % CO_2. The supernatants that were harvested were either used immediately in an ELISA or stored at -20°C until further use.

3.3 Medium stimulation

As a control test to determine if the mitogens bind with the capture and/or detecting antibodies, thus giving false positive results, medium (RPMI + L-glutamine) samples were stimulated with the same mitogens and concentrations as the PBMC/WB samples and incubated and stored under the same conditions.

3.4 Interferon-gamma ELISA

To determine the optimal concentration of the capture antibody (1D11), two concentrations (0.5 μg/well & 0.25 μg/well) were used in the first ELISA. For the following ELISAs, a concentration of 0.25μg/well 1D11 was used. Polysorb 96-well ELISA plates (NUNC) were coated with capture antibody 1D11 (50 μl/well), and the plates were incubated at 4 °C overnight. The next day the buffer was discarded and the wells were blocked with 100 μl block buffer (2% milk powder (MP) in PBS). Another block buffer (2% BSA in PBS) was tested to see if there was a difference in background noise between the two block buffers. The plates were incubated for 1 hr at 37 °C. After incubation, the plates were washed five times using an ELISA washer with distilled water as the wash buffer. To determine the ideal concentration for the positive control, recombinant IFN-γ (horse and rhino) was added in 12 dilutions (with Block Buffer 2 (BB2): 2% MP (or 2% BSA) and 0.1% Tween 20 in PBS), starting with a concentration of 2 μg/well. For the negative control 50 μl of BB2 was added. The stimulated whole blood and PBMC samples together with the stimulated medium as a control were also tested by using 50 μl of the supernatant/medium per well. In all ELISAs the recombinant antigen served as the positive control. As a negative control, BB2 was also used. The plates were incubated for 1 hr at 37°C. The plates were washed five times using the ELISA washer with wash buffer 0.1% Tween 20 in distilled water. After washing the plate, 50 μl of polyclonal chicken anti-IFN-γ antibody (IgY), diluted in BB2, was added to the wells at a concentration of 1 μg/ml. After the addition of IgY, the plates were incubated for 1 hr at 37°C. The plates were washed five times with wash buffer. The conjugate, rabbit polyclonal to chicken IgY horse radish peroxidase (HRP) (Abcam), was added to the wells (50 μl/well) in a 1:3000 dilution in BB2. The plates were incubated for 1 hr at 37 °C. The wash step was repeated

after which, the substrate ortho-phenylenediamine (OPD) (5 mg OPD, 2.5 µl H_2O_2 in 5 ml citrate buffer) (50 µl) was added to the wells. The plates were incubated for 30 min at room temperature. The reaction was stopped with stop solution (2N H_2SO_4) (50 µl/well). After 5 min, the plates were read with an ELISA reader at an optical density (OD) of 492 nm and the results were then analysed.

4. Results

4.1 Optimal coating antibody and recombinant IFN-γ concentration

To determine the detection limit of the ELISA the first experiment made use of two different concentrations of capture antibody (0.5 µg/well & 0.25 µg/well). A 2x's dilution series for the recombinant horse and rhinoceros IFN-γ (rEqIFN-γ & rRhIFN-γ) were used, starting with 2 µg/well and ending with 0.0625 µg/well (Fig. 1 & Fig. 2). The last well served as the negative control as no recombinant antigen was added. These results indicated that a further dilution of the protein was required to determine the detection limit. The use of either MP or BSA as the block buffer in this ELISA indicated that the use of MP gave lower background signals compared to BSA when the negative control sample was compared. The second ELISA was performed using 0.25 µg/well of capture antibody (Fig. 3 & Fig. 4).

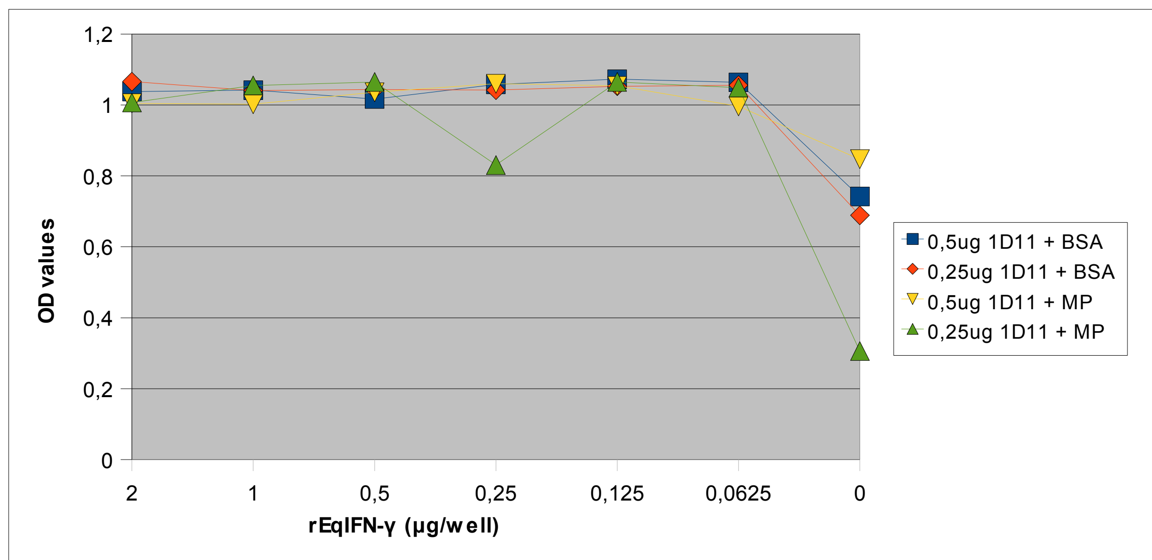

Figure 1 Dilution series of rEqIFN-γ. Capture antibody (1D11) concentrations and blocking buffers are indicated in the legend

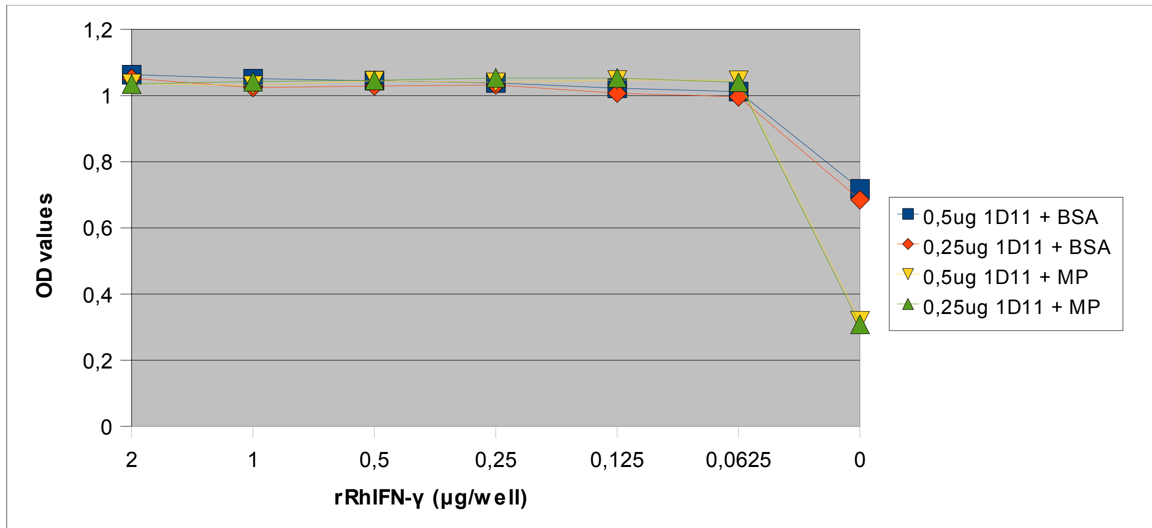

Figure 2 Dilution series of rRhIFN-γ. Capture antibody (1D11) concentrations and blocking buffers are indicated in the legend.

The results of the rEqIFN-γ ELISA (Fig. 3) indicate that when using BSA as the block buffer the detection limit of the ELISA is 0.03 µg/well and when using MP as the block buffer the detection limit of the ELISA is 0.125 µg/ml. However, the use of milk powder does provide a lower background signal.

For the rRhIFN-γ ELISA (Fig. 4) a concentration of 0.004 µg/well of recombinant protein could be detected with the use of BSA as the blocking buffer. When MP was used the optimal concentration of rRhIFN-γ that could be detected was 0.015 µg/well. Overall the use of MP reduced the background signal to a higher degree when compared to the use of BSA in the same ELISA. The results depicted in Fig. 3 & Fig. 4 are summarized in Table 1 below.

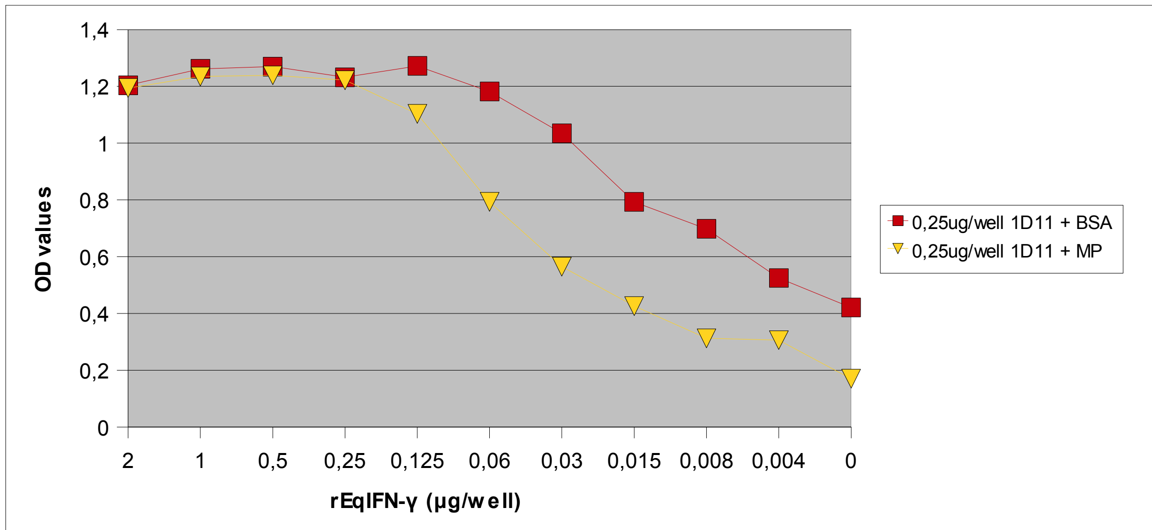

Figure 3 Dilution series of rEqIFN-γ. Capture antibody (1D11) concentrations and blocking buffers used are indicated in the legend.

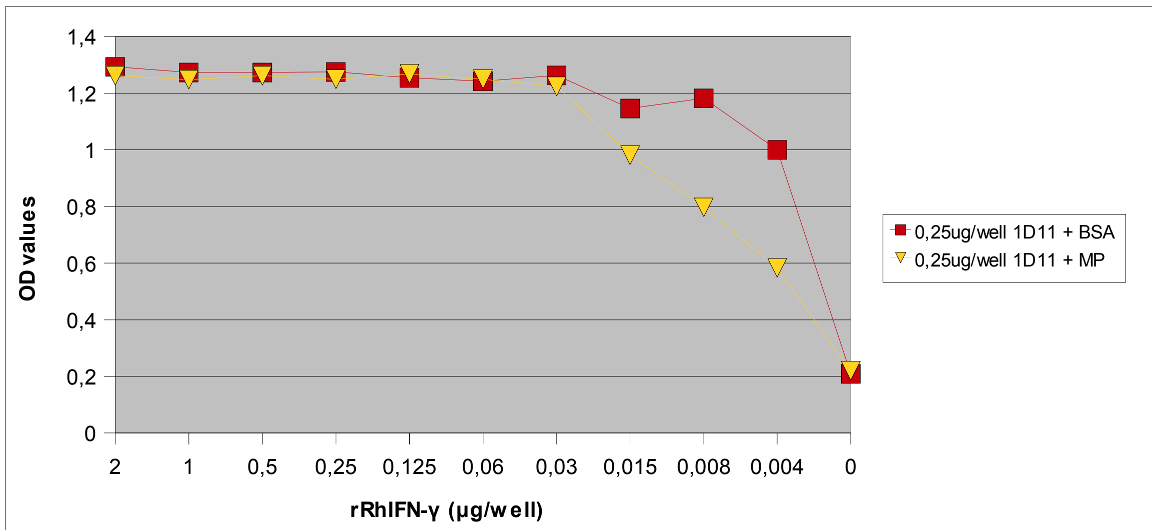

Figure 4 Dilution series of rRhIFN-γ. Capture antibody concentrations and blocking buffers used are indicated in the legend.

Table 1 Summary of ELISA using different block buffers

1D11	*Buffer*	*rRhIFN-γ*		*0 µg/ml*	*Signal*	*rEqIFN-γ*		*0 µg/ml*	*Signal*
µg/well		**µg/well**	**OD**	**OD**	**OD**	**µg/well**	**OD**	**OD**	**OD**
0.25	BSA	0.004	1	0.2	**0.8**	0.03	1	0.4	**0.6**
0.25	MP	0.004	0.3	0.2	**0.1**	0.03	0.6	0.3	**0.3**
0.25	BSA	0.015	1.1	0.2	**0.9**	0.125	1.3	0.4	**0.9**
0.25	MP	0.015	1	0.2	**0.8**	0.125	1.1	0.3	**0.8**

4.2 Detection of native IFN-γ

Supernatant harvested at three time intervals from whole blood, PBMCs and medium samples, stimulated with varying concentrations of different mitogens, were tested in the ELISA optimized in section 4.1. A 1D11 concentration of 0.25 μg/well was used for coating. For the positive (+ve) control, a concentration of 0.125 μg/well of rEqIFN-γ was used. For the negative (-ve) control (no antigen added) BB2 was used. The results from the whole blood samples (Fig. 5) show that stimulation with PWM gives the highest OD values at all stimulation intervals. The PHA (10 μg/ml) sample at 72 hrs also indicates a strong detection signal. Use of PHA at a concentration of 2.5 μg/ml and the signals provided by the SEB stimulated samples are much lower. The use of Con A does not indicate detection of native EqIFN-γ. Medium (no cells) stimulated with mitogens tested in the ELISA (Fig. 6) show that all mitogens give low OD values, near to the negative control, except Con A 10 μg that seems to give signals twice as high as the negative control signal. The signals, obtained for the medium control from the 72 hrs incubated samples, are slightly higher for all the mitogens.

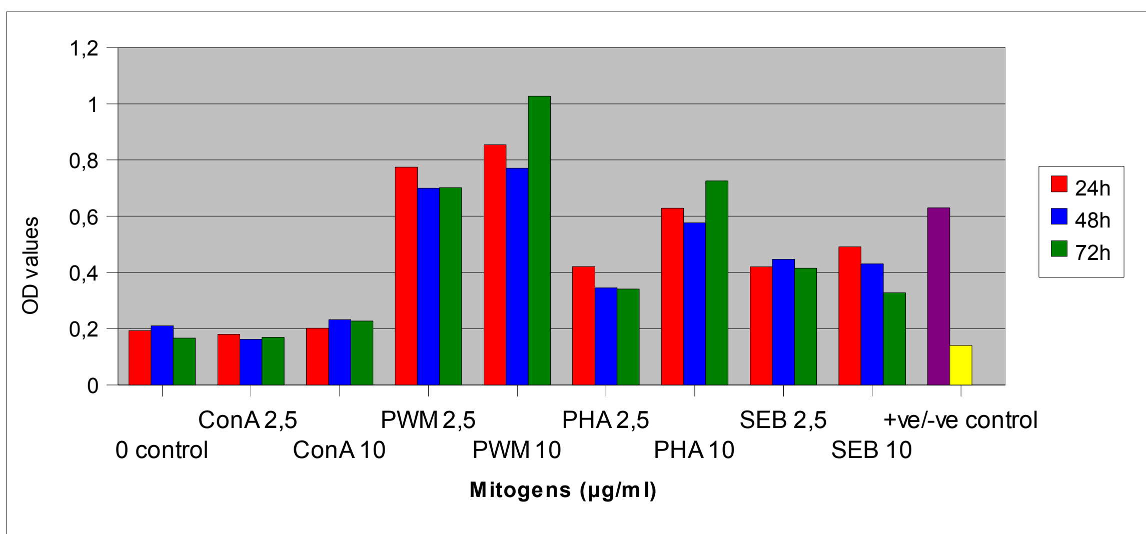

Figure 5 Whole blood stimulated with 4 mitogens in different concentrations incubated at 3 different times.

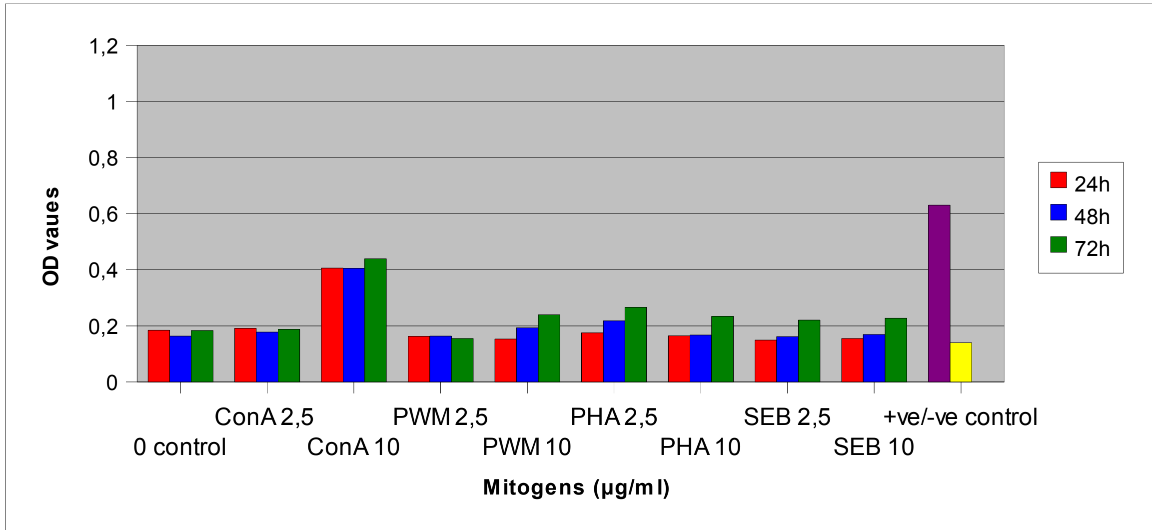

Figure 6 Medium (no cells) stimulated with 4 mitogens in different concentrations.

For the PBMCs (Fig. 7), the 0 control values are higher than those from the WB and medium samples. High values are obtained with all mitogens, except for SEB 72 hrs, which indicates no detection of native EqIFN-γ. As seen in all three figures, the signals do not increase when a longer incubation time is used.

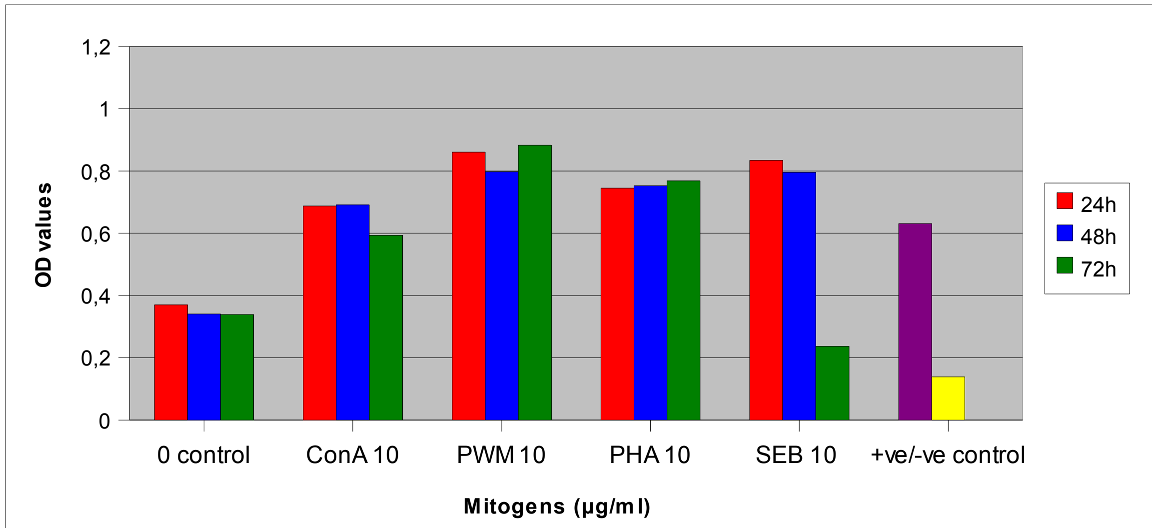

Figure 7 PBMCs stimulated with 4 mitogens incubated at 3 different incubation times.

4.3 The use of mitogens PWM and PHA at different concentrations and incubation times

4.3.1 Whole blood and medium

An ELISA was performed using supernatant harvested from whole blood and medium samples, stimulated with mitogens PWM and PHA at different concentrations and incubated for 24 hrs. The supernatants were used after storing them at -20°C for one day. For coating, a concentration of 0.25 µg/well 1D11 was used. A concentration of 0.25 µg/well rEqIFN-γ was used for the positive control. The results of the ELISA indicate detection of native IFN-γ from varying concentrations of PWM stimulated whole blood (Fig. 8). This is also the result when using different concentrations of PHA (Fig. 9), although background signals are lower with the samples stimulated with PWM.

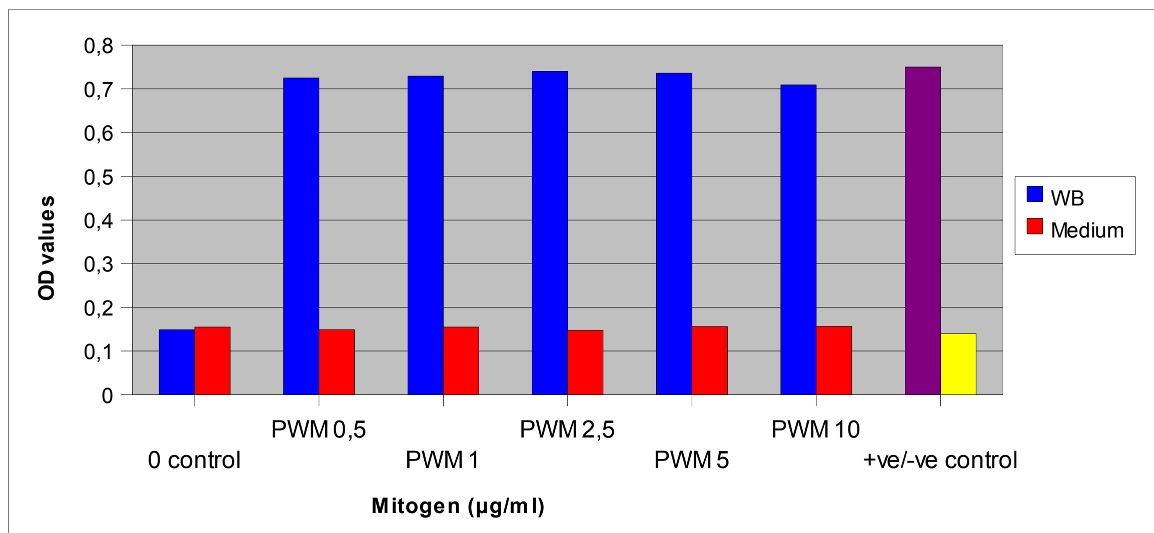

Figure 8 Whole blood and medium stimulated with different concentrations of PWM after 24 hrs incubation.

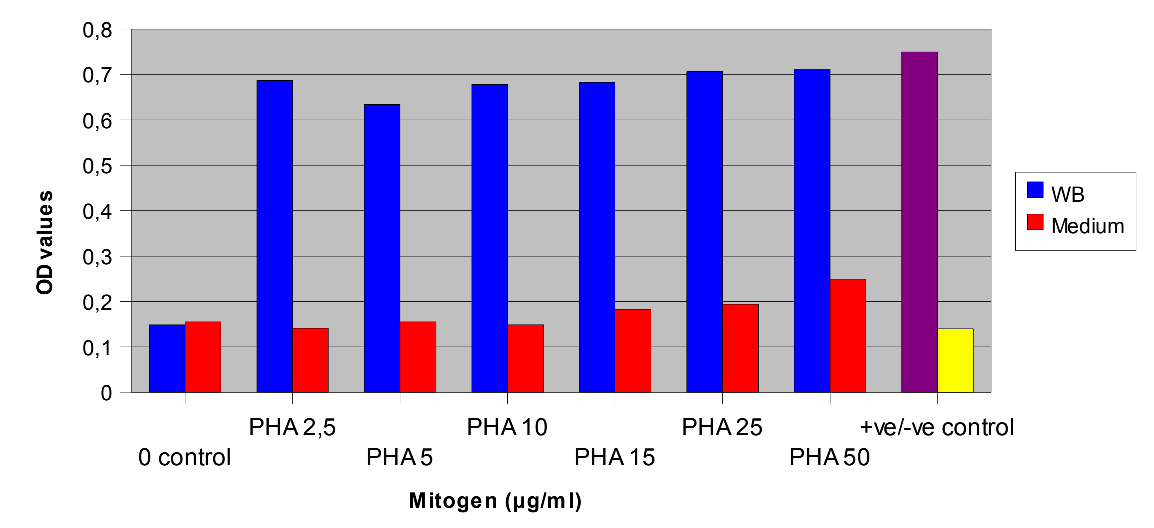

Figure 9 Whole blood and medium stimulated with different concentrations of PHA after 24 hrs incubation.

4.3.2 PBMC and medium

After a 24 hr incubation, freshly harvested supernatants from PBMCs and medium stimulated with mitogens PWM and PHA (See 4.3.1), were tested in an ELISA coated with 0.25 µg/well 1D11. A concentration of 0.25 µg/well rEqIFN-γ was used for the positive control. Detection of native IFN-γ from stimulated PBMCs, using both mitogens, is achieved (Fig. 10 & Fig. 11). As seen in the whole blood ELISAs (Fig. 8 & Fig. 9), the signals obtained from the stimulated medium increase slightly with an increasing concentration of the mitogens. The signals for both PWM and PHA are almost similar irrespective of whether WB or PBMC samples are stimulated.

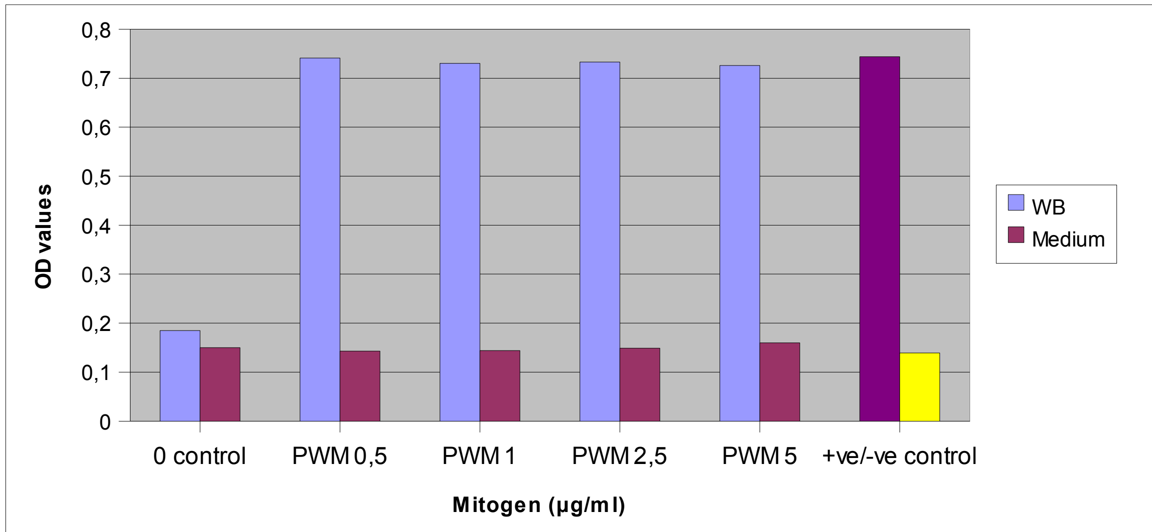

Figure 10 PBMC and medium stimulated with PWM in different concentrations, after 24 hrs incubation.

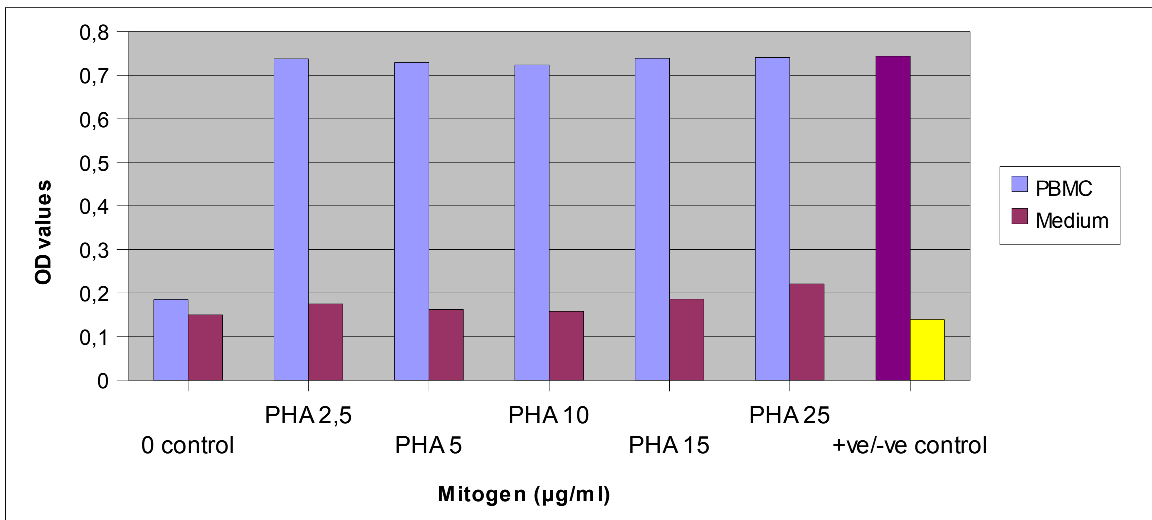

Figure 11 PBMC and medium stimulated with mitogen PHA in different concentrations, after 24 hrs incubation.

4.3.3 Different incubation times

An ELISA was performed with the supernatants harvested from stimulated PBMC and medium samples after 24, 48 and 72 hrs incubation. The samples were used after one freeze-thaw cycle. Positive control (0.25 µg/well rEqIFN-γ) and negative control OD values in this ELISA were 0.771 and 0.141 respectively. There is no difference in OD values between the different incubation times (Fig. 12). However, there is a slight increase in background.

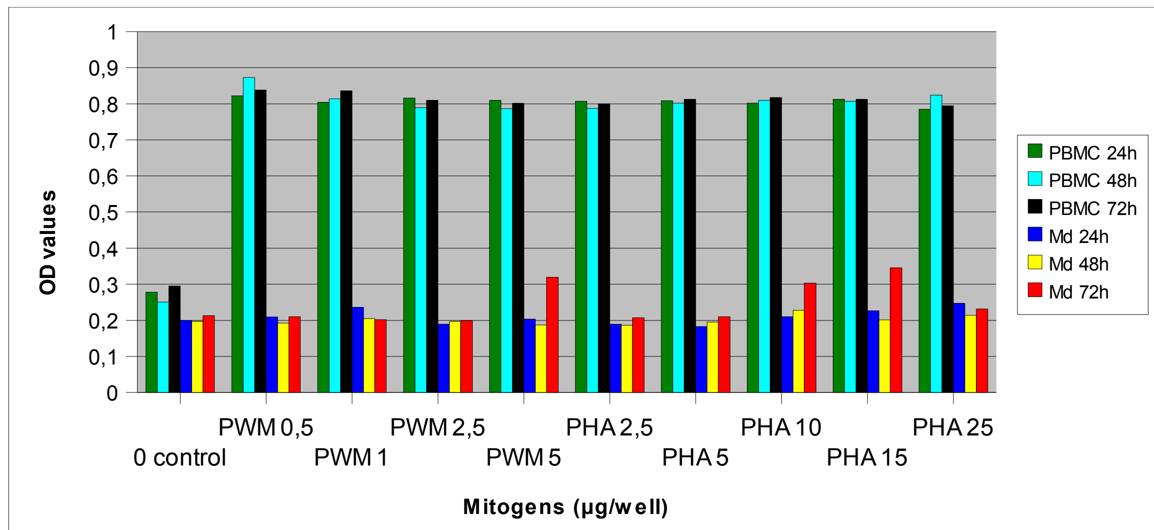

Figure 12 PBMC and medium samples stimulated with different concentrations of PWM and PHA. Incubation times are indicated in the legend.

5. Discussion

The aim of this study was to optimize the IFN-γ assay developed for rhinoceroses (Morar *et al.* 2007). Horse blood was used for this study because blood samples from rhinoceroses were not available during the period that this research project was performed. Horse blood was the preferred alternative as the IFN-γ sequences of these two species show a 90% homology at the amino acid level (Morar *et al.* 2007).

Several optimization experiments were performed. The first of which was to determine the optimal concentration of the capture antibody and the detection limit of both horse and rhinoceros recombinant IFN-γ. This condition would then serve as the positive control in all subsequent ELISAs that were performed. Monoclonal capture antibody 1D11, produced against rRhIFN- γ, and polyclonal detecting antibody IgY were used in the capture ELISA, because this combination of antibodies was proven to be successful in the detection of both recombinant and native white RhIFN-γ (Morar *et al.* 2007).

Two concentrations of 1D11 were tested (0.25 μg/well & 0.5 μg/well) with a dilution series of recombinant antigen, starting with 2 μg rIFN-γ/well (horse & rhino). No difference was found between the 1D11 concentration of 0.5 μg/well and 0.25 μg/well and therefore a concentration of 0.25 μg/well of 1D11 was used as the required concentration in these ELISAs. An IgY concentration of 1 μg/ml was used in the ELISAs, because this was found to be the optimal concentration in previous studies (Morar 2009).

For rEqIFN-γ, a concentration of 0.125 μg/well was found to be the lowest concentration at which a positive signal was obtained. For rRhIFN-γ, the lowest concentration was 0.015 μg/well.

To determine the ideal block buffer for the ELISA, two different blocking buffers (2% MP in PBS and 2% BSA in PBS) were tested. The 2% MP in PBS gave the best results; therefore this blocking buffer was used in ELISAs performed in this project.

Supernatants from both whole blood (WB) and peripheral blood mononuclear cells (PBMC) were tested in the ELISA. In previous experiments, it was shown that the results were more pronounced using PBMCs. However, isolating these cells is a very time consuming process, therefore whole blood was processed. No differences between the signals were found in several of the experiments that were performed, which indicated that whole blood samples can be used for the assay. This was an advantage as it reduced the sample processing time.

Blood samples were stimulated with a mitogen, to test for the detection of cytokine IFN-γ. To establish that the ELISA used in this study could detect recombinant and species specific (native) IFN-γ, both recombinant IFN-γ and mitogen stimulated samples were used.

In previous experiments, using the IFN-γ assay, it was found that the results were inconsistent and not repeatable (Maas 2008). The use of Con A as a mitogen was questioned and found that this mitogen has an affinity for binding to glycopeptides (Sparbier *et al.* 2006). It was also found that Con A present in the cell supernatant was binding to the antibodies used for capture and detection in the assay. To overcome this problem, this study made use of other mitogens, like PWM, SEB and PHA.

In addition to the blood samples, controls which included only medium (RPMI + L-glutamine) were stimulated with the same mitogens; to test if the mitogens would bind with the capture and/or detecting antibodies, thus indicating false detection signals.

The experiments performed in this study using all four mitogens showed that mitogen PWM gave the best signals, even when used at low concentrations. Mitogen PHA also looked promising, so both mitogens were tested again in whole blood and PBMCs at varying concentrations to determine which of the mitogens would be the optimal one to be used for the production and subsequent detection of native IFN-γ in the assay. Both mitogens showed the same high OD values in all concentrations tested. The results of the ConA stimulated samples were consistent with previous experiments (Maas 2008). The

signals obtained were low and inconsistent in the different ELISAs performed in this study. The signals obtained with SEB stimulated samples were also low and inconsistent, especially in the whole blood samples.

The ELISAs tested with the medium samples showed that all mitogens gave low signals, near to those of the negative control values, exept for ConA 10 µg. This is also consistent with earlier findings concerning the use of ConA (Sparbier *et al* 2006, Maas 2008).

The use of mitogens differs for different species in studies of the IFN-γ assay. Dalley *et al.* (2004) showed that mitogen PWM was the optimal mitogen to be used in the IFN-γ assay developed for badgers. Rhodes *et al.* (2008) used the mitogen phorbol myristate acetate plus calcium ionophore (PMA/Ca) in her ELISA for cats (*Felis domesticus*). Mitogen PHA was used in the development of a human IFN-γ assay (Desem & Jones 1998)

For the stimulated whole blood and PBMCs, different incubation periods (24, 48 and 72 hrs) were tested to see if longer incubation of the samples would result in higher IFN-γ production. The mitogens tested were ConA, PWM, PHA and SEB in the concentrations of 2.5 µg/ml and 10 µg/ml. It was found that the signal does not improve when blood samples from horses were incubated longer than 24 hrs (48 hrs or 72 hrs). The ideal incubation time is 24 hrs before the supernatant is tested in the ELISA for the production of IFN-γ. This correlates with other studies where it is seen that optimal incubation times differ for different species. The incubation time used by Morar *et al.* (2007) in the IFN-γ assay for rhinoceroses was 18-24 hours. Rhodes *et al.* (2008) used an incubation time of 4 days for their ELISA prototype for felines. Bosward *et al.* (2010) found that maximum IFN-γ synthesis occurred in sheep with an incubation period of 48 hrs. In the assay developed for badgers (Dalley *et al*. 2008), an incubation period of 16-24 hrs was used. Schiller *et al*. (2009) used an incubation time of 24 hrs in an optimization study for the Bovigam™ ELISA for cattle.

It was found that the strength of the signals from the recombinant antigen (positive controls) dropped in course of time. A higher concentration of rEqIFNγ (0.25 μg/well instead of 0.125 μg/well) was therefore used as a positive control in the ELISAs described in results section 4.3 and further. The drop in signal could be due to the frequent freeze-thaw of the reagents (antigen and IgY). To overcome this, the reagents were aliquoted into smaller volumes to reduce the number of freeze-thaw cycles.

To test the viability of supernatant after one freeze-thaw cycle (-20°), an ELISA was performed on freshly harvested samples and compared to samples that were frozen and thawed. No difference was seen between the samples after one freeze-thaw cycle. It could be that more than one freeze-thaw cycle contributes to a decrease in signal. This, however, has yet to be determined as it was not tested in this study due to time constraints. Schiller *et al.* (2009) showed a high level of stability in bovine IFN-γ produced in whole blood culture. The responses from IFN-γ stored at -80°C with up to five freeze-thaw cycles did not differ from the results obtained with freshly harvested plasma.

6. Conclusion

Several optimization experiments for the rhinoceros IFN-γ ELISA have been performed in this study. Unfortunately, rhinoceros blood samples could not be obtained during the period of this research, so only horse blood could be used. The next step will be to test native rhinoceros samples in the IFN-γ assay that has been optimized in this study. Although this study only forms a basis for the optimization for further work using rhinoceros blood samples, it has the versatility of being used as a diagnostic test for horses. In addition this form of the ELISA is not only limited to diagnosing *M. bovis* infections in horses and eventually rhinoceros but can be used for diagnosing other diseases in which IFN-γ plays a role in mediating a cellular immune response to an infection.

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