Transboundary and Emerging Diseases

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ORIGINAL ARTICLE

Towards Establishing a Rhinoceros-Specific Interferon-Gamma (IFN-γ) Assay for Diagnosis of Tuberculosis

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Summary

Mycobacterium bovis is the causal agent of bovine tuberculosis (BTB), with a diverse host range, extending from livestock to domestic and captive wild animals as well as free-ranging wildlife species. In South Africa, BTB is endemic in the Kruger National Park (KNP) and the Hluluwe iMfolozi National Park (HiP), where the high prevalence of *M. bovis* infections in buffalo herds has led to infection of a number of wildlife species. This has raised concerns about the spill-over into the rhinoceros population, a species known to be susceptible to both *M. bovis* and *Mycobacterium tuberculosis*, jeopardizing breeding and relocation projects that serve to conserve and protect this species. In view of the advantages of the interferon-gamma (IFN-γ) assay in the diagnosis of BTB in a variety of species worldwide, such an assay has been developed for rhinoceroses by Morar and co-workers in 2007. In this study, this assay was optimized using recombinant eukaryotic rhinoceros IFN-γ and the lower detection limit was calculated to be 0.5 ng/ml. Subsequently, assessing the detection of native rhinoceros IFN-γ protein in whole-blood samples revealed stimulation with each of the mitogens: pokeweed (PWM), phytohaemagglutinin (PHA) & phorbol 12-myristate 13-acetate and calcium ionophore (PMA/CaI), though most prominently with the latter two. In addition, samples collected from 52 clinically healthy rhinoceroses, of presumed negative BTB status, from two different areas in South Africa were used to determine the cut-off value for a negative test result. This was calculated to be 0.10 ($OD_{490\ nm}$) and as determined in this study is a preliminary recommendation based on IFN-γ responses observed in samples from BTB-free rhinoceroses only.

Introduction

South Africa has the largest population of white rhinoceroses (*Ceratotherium simum*) in the world and is home to a significant number of black rhinoceroses (*Diceros bicornis*). These animals provide an important source of income to national parks, such as the Kruger National Park (KNP), as they are a major tourist attraction as well as from direct sales both nationally and internationally, contributing significantly to the improvement of the conservation status for both white and black rhinoceroses in Africa. In South Africa, the first case of a mycobacteriosis in a black rhinoceros was diagnosed in 1970 in the Hluhluwe-iMfolozi Park (HiP), Kwazulu Natal province (Keep and Basson, 1973). Later in the 1990s, also in the HiP area, the histopathological examination of lymph node specimens from a black

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rhinoceros indicated lesions typical of those caused by a mycobacterial infection. The diagnosis remained unconfirmed as culture results were not available (Kriek, N. Faculty of Veterinary, Science, University of Pretoria, personal communication, 2012). More recently, bovine tuberculosis (BTB) caused by *Mycobacterium bovis* was reported in a black rhinoceros (*Diceros bicornis minor*) (Espie et al., 2009). This, along with other reports of tuberculosis in captive rhinoceroses (Mann et al., 1981; Dalovisio et al., 1992; Stetter et al., 1995), not only confirms that these animals are susceptible to infection by *M. bovis,* but also illustrates the need for diagnostic assays able to support the monitoring of the BTB-free status of these animals. Conservation efforts would also greatly benefit from an early warning system should BTB enter the rhinoceros population in wildlife reserves. While tests traditionally available for diagnosing BTB include microscopy and bacterial culture techniques, as well as the intradermal tuberculin skin test (IDT) (Monaghan et al., 1994), these are of little value for screening purposes in rhinoceroses. The intradermal skin test is not practical due to difficulties in defining suitable injection sites in pachyderm animals, and culture techniques, although presumably the most reliable and specific, are performed on either post-mortem or clinical specimens, which require invasive sampling and results are obtained only after 6–8 weeks. The lack of BTB diagnostic tools for use in rhinoceroses led to the development of a rhinoceros-specific IFN-γ assay (Morar et al., 2007), which allows measurement of cell-mediated immunity (CMI), because immune responses (including protective immunity) against mycobacteria are primarily CMI-based prior to the onset of clinical signs (Andersen et al., 2000; Pollock et al., 2001; Pollock and Neill, 2002). Although a number of IFN-γ tests have been developed for various wildlife species (Garcia et al., 2004; Maas et al., 2012), validation of these types of tests remains a challenge, especially when BTB occurs in few instances or the species affected is rare and or valuable (Cousins and Florisson, 2005).

The aim of this study was to characterize previously undetermined parameters of the analytical test performance, namely the lower limit of detection (LOD) of recombinant RhIFN-γ (analytical sensitivity) as well as selection of a mitogen as positive control stimulus for RhIFN-γ production in whole-blood cultures. The study furthermore aimed at determining the test specificity of the IFN-γ assay in whole-blood samples of healthy rhinoceroses with a presumed negative BTB status, considering the stringent requirements for test validation as set out by the World Organisation for Animal Health (OIE) (OIE Terrestrial Manual, 2012). The cut-off for negative test results, as determined in this study, is a preliminary recommendation based on observed IFN-γ responses in BTB-free rhinoceroses only and will be adapted once infected animals are available to determine specificity in combination with test sensitivity.

Materials and Methods

Sample collection

Blood samples were collected in heparin vacutainer tubes from a total of 75 clinically healthy white rhinoceroses (*Ceratotherium simum*) from three different areas in South Africa, which include the KNP ($n = 23$) in Mpumalanga province, one game reserve in North West province ($n = 9$) and one game farm in Gauteng province ($n = 43$) (Table 1). Unlike the KNP, where BTB is endemic, the reserve and game farm have no known history of BTB. The heparinized blood was kept at ambient temperature during transportation to the laboratory and processed within 8 h after collection.

Animal ethics

Animals were not darted specifically for the collection of samples for the project, but samples were collected when animals were immobilized for purposes of dehorning and or capture for export and translocation. The project was approved by both the Research Committee and the Animal Use and Care Committee (AUCC) of the University of Pretoria.

Optimization of analytical sensitivity of the IFN-γ assay

Capture ELISA

The rhinoceros-specific IFN-γ ELISA, which was initially developed with prokaryotic rRhIFN-γ (Morar et al., 2007), was optimized for the detection of glycosylated rRhIFN-γ (grRhIFN-γ, protein expressed using an eukaryotic system – U-Protein Express BV, The Netherlands), assumed to be more similar to the native protein than the previously used *E. coli*-derived rRhIFN-γ. Optimization was performed using a checkerboard (titration) ELISA to determine the optimal concentrations of both the capture and detecting antibodies. This was followed by determining the detection limit of grRhIFN-γ. The optimized protocol was as follows: Polysorb ELISA (Nunc) plates were coated with capture antibody 1D11 (Morar et al., 2007) in carbonate

Table 1. Number of animals sampled from BTB-free and BTB-endemic areas

Area	Number
Game reserve (BTB-free)	9
Game farm (BTB-free)	43
KNP (BTB-endemic)	23
Total	75

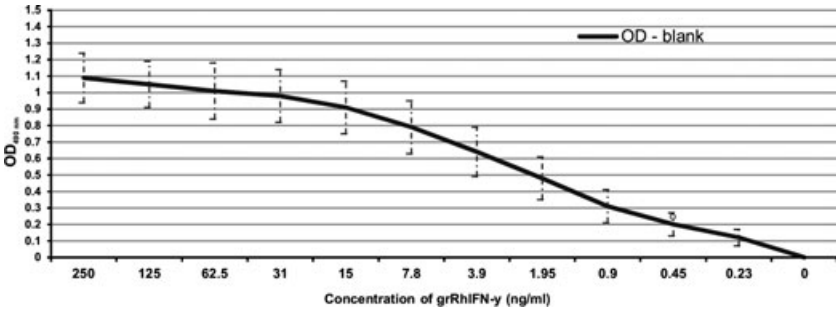

Fig. 1. Averaged IFN-γ calibration curves (n = 8) expressed as $OD_{490\ nm}$. The line represents the $OD_{490\ nm}$ after subtraction of the blank sample. Error bars indicate the standard deviations. The lower detection limit of the assay was 0.5 ng/ml grRhIFN-y, based on the average ($OD_{490\ nm}$) of the blank samples + 2*standard deviation of the blank samples [0.13 + 2 × 0.05 = 0.23], obtained using $OD_{490\ nm}$ values before the nil subtraction.

bicarbonate buffer (pH 9.6) at a concentration of 5 μg/ml and incubated at 4°C overnight. The next day the coating buffer was discarded, and 300 μl of 4% blocking buffer (fat-free milk powder in PBS) was added to each well. The plates were then incubated at 37°C on a shaker for 30 min. After this step, the blocking buffer was discarded and the plates were washed 3 times with distilled water (dH_2O), and the stored plasma was added to the wells. Two-times serial dilutions of grRhIFN-γ (U-Protein Express BV, The Netherlands) were used to produce a calibration curve for each ELISA plate used (Fig. 1). The plate was then incubated at 37°C on a shaker for 1 h. The plate was washed 3 times with wash buffer (dH_2O containing 0.1% Tween 20) followed by the addition of anti-rhinoceros IFN-γ polyclonal IgY (Morar et al., 2007) at a final concentration of 1 μg/ml (50 μl/well). Incubation and wash steps with wash buffer were repeated as mentioned previously, followed by the addition of a 1:3000 dilution of the conjugate (rabbit α-chicken HRP labelled; Abcam) at 50 μl/well to all wells on the ELISA plate and incubated for 1 h at 37°C on a shaker. After the incubation step, the plate was washed 3 times with wash buffer and twice with dH_2O to remove any remaining detergent (Tween 20) from the wells, and substrate OPD (SIGMAFAST™ OPD), prepared according to manufacturer's instructions, was added to each well at a final volume of 200 μl/well. The colour reaction was stopped after 30 min by addition of 2 M H_2SO_4 (50 μl/well), and plates were read at an optical density (OD) of 490 nm to measure the absorbance readouts.

Mitogenic stimulation of blood samples

For initial optimization, blood from 4 rhinoceroses in the KNP was collected in heparin vacutainer tubes. Samples were processed within 6–8 h after collection and stimulated with different mitogens (10 μg/ml – pokeweed mitogen [PWM], 10 μg/ml phytohaemagglutinin [PHA] & 100 ng/ml phorbol 12-myristate 13-acetate and 2 μg/ml calcium ionophore [PMA/CaI]) for 48 and 72 h at 37°C, in a CO_2 incubator. A negative control comprising the blood sample mixed with medium (nil stimulation) instead of a mitogen was also included. After incubation, supernatants were harvested from all samples by centrifugation at 1088 ***g*** (Eppendorf centrifuge 5430 R) for 5 min. The supernatant was either used directly in the ELISA or stored at −80°C until use.

Whole-blood culture

Heparinized blood of 52 rhinoceroses from BTB-free areas and 23 rhinoceroses from the BTB-endemic KNP was dispensed into 2-ml eppendorf tubes at a final volume of 1.5 ml/tube and stimulated with mycobacterial antigens, namely avian (20 μg/ml) and bovine (20 μg/ml) PPDs and incubated at 37°C in 5% CO_2 for 48 h. A negative control, addition of medium to the blood sample, and a positive control, PMA (100 ng/ml) & CaI (2 μg/ml) stimulated sample, were included in this step to measure the cells' competency to respond to stimulation. After the incubation time period of 48 h elapsed, the plasma was harvested from the samples by centrifugation at 1088 g for 5 min and stored at −80°C until use in the rhinoceros-specific IFN-γ capture ELISA.

Data analysis

Determining the lower limit of detection (LOD)

The LOD was based on the concentration of grRhIFN-γ that gave an $OD_{490\ nm}$ reading higher than the average (μ) $OD_{490\ nm}$ of the negative control/blank plus two times the standard deviation (σ) [$\mu_{blank} + 2*\sigma$]. For all samples, the readings obtained were corrected in relation to nil stimulated samples. Supernatants from the stimulated whole-blood samples were assayed in duplicate, and the IFN-γ responses were expressed as $OD_{490\ nm}$ values (average of the duplicates), and in concentration levels (ng/ml) of IFN-γ detected. The IFN-γ concentration levels for all samples

were calculated using the calibration curve run on the same ELISA plate as the samples. If the response of any of the samples stimulated with mitogen (PMA/CaI) was below the $OD_{490\ nm}$ reading of the mean (μ) mitogen stimulated samples (n = 75) minus two times the standard deviation (σ) ($OD_{mitogen} < \mu_{mitogen} - 2*\sigma_{mitogen}$), they were excluded from the panel of samples used to determine a cut-off for a negative test result in this study.

Cut-off value for a negative test result

The test performance (OIE Terrestrial Manual, 2012) of the IFN-γ assay was calculated as the proportion of samples from known uninfected animals (BTB-free areas) by measuring the IFN-γ responses to both avian (environmental control) and bovine PPD stimulation. To determine the cut-off value for a negative test result in this study, the following equation $\mu(OD_{bov\ -\ nil}) + 2*\sigma_{standard\ deviation}(OD_{bov\ -\ nil})$ was used.

Results

Optimization of analytical sensitivity of the IFN-γ assay

Capture ELISA

Capture and detecting antibodies were tested by checkerboard titration to determine the concentration ranges for the detection of glycosylated recombinant protein, grRhIFN-γ. Averaged calibration curves produced in all ELISA plates (n = 8) and used as the internal positive controls in this experiment are shown in Fig. 1. The LOD was calculated to be 0.5 ng/ml (Fig. 1) grRhIFN-γ, based on the average ($\mu OD_{490\ nm}$) of the blank samples + 2*σ [μOD_{blank} 0.13 + (2*σOD_{blank} 0.05) = 0.23] prior to the subtraction of the blank $OD_{490\ nm}$ values from the initial $OD_{490\ nm}$ values obtained. The aim to select a mitogen for use as a positive control for the potency of production of native RhIFN-γ in whole-blood cultures was determined by the strongest and most consistent $OD_{490\ nm}$ responses in repeat testing of the same sample. In this regard, PWM and PMA/CaI, respectively, performed better than PHA in eliciting the production of native IFN-γ (results not shown) as detected by the RhIFN-γ ELISA. These mitogens were used as the positive control stimuli in subsequent whole-blood cultures. Furthermore, highest quantities of IFN-γ were found to be produced in the incubation interval of 48 h, which was used for all subsequent assays.

Assessment of the test specificity of the IFN-γ assay

The production of IFN-γ after nil stimulation, mitogen stimulation and stimulation with avian and bovine PPDs was measured in whole-blood cultures of 75 clinically healthy rhinoceroses from three different populations in South Africa. All animals from both the BTB-free and BTB-endemic areas, except one, had $OD_{490\ nm}$ readings higher than 0.42 [$\mu_{mitogen} - 2*\sigma_{mitogen}$]. No significant IFN-γ (ng/ml) production was found after antigenic stimulation with bovine and avian PPDs (Fig. 3) in comparison with the negative controls. For the BTB-free group (n = 51), responses ($OD_{490\ nm}$) to avian PPD stimulation ranged from 0 to 0.16 after nil subtraction (Fig. 2). Responses ($OD_{490\ nm}$) to bovine PPD stimulations ranged from 0 to 0.19 after subtraction of nil values (Fig. 2).

For the BTB-free group (n = 51), the μOD_{bov} calculated value resulting from stimulation with bovine PPD was 0.02. The cut-off value for a positive test result was calculated to be OD_{bov} 0.1 (0.02 + 2 × 0.04) after subtracting the nil value. Based on this, three animals Rh30 (OD_{bov} = 0.11; OD_{av} = 0.04), Rh33 (OD_{bov} = 0.19; OD_{av} = 0.09) and Rh48 (OD_{bov} = 0.13; OD_{av} = 0.16) showed elevated responses to bovine PPD stimulations and should be considered as false positives. Using the cut-off values defined

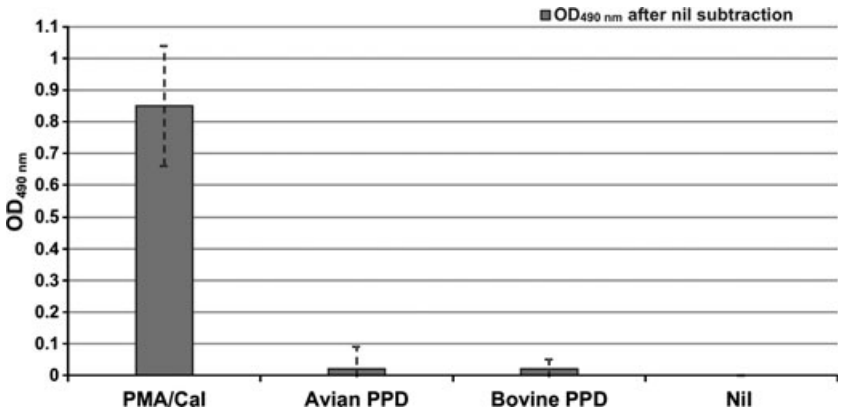

Fig. 2. Averaged IFN-γ responses after 48 h stimulation from whole-blood samples of 51 rhinoceroses, expressed as $OD_{490\ nm}$ values after subtraction of nil values for all conditions tested, from BTB-free areas only. Error bars indicate the standard deviations.

here, preliminary calculations indicate a test specificity of 94% ($^{48}/_{51} \times 100$).

Discussion

Specific conditions related to the test performance of the assay were determined and assessed in this study. These included the selection of a suitable mitogen for use as a positive control, incubation time of samples, and optimal concentrations of detecting and capture antibodies as well as the detection limit of the recombinant antigen. The LOD of the grRhIFN-γ in the optimized capture ELISA is 0.5 ng/ml. This may be improved in future using the detection antiserum IgY after affinity purification to grRhIFN-γ to reduce background staining in combination with a stronger conjugate and a more sensitive substrate to increase the specific signal. Pokeweed mitogen and PMA/CaI were identified as the best performing mitogens for use as positive controls in stimulation of rhinoceros whole-blood cultures. The mitogen combination of PMA and CaI has also been used by Rhodes et al., 2008 and Maas et al., 2012 as a positive control in their respective feline and lion IFN-γ assays.

Interferon-γ assays conducted after stimulation of whole blood from 75 clinically healthy rhinoceroses indicated that a majority ($n = 74$) of the animals produced detectable levels of IFN-γ following stimulation with mitogen. Only one sample did not produce favourable IFN-γ responses after mitogen stimulation. When considering IFN-γ responsiveness towards the bovine PPD (*M. bovis*-derived antigen), the vast majority of the 51 rhinoceroses did not show notable responses (Figs 2 and 3) compared with nil-stimulated samples, with the exception of three animals which yielded $OD_{490\ nm}$ values above the cut-off value (OD_{bov} 0.10), and which were hence classified as false-positive reactors. Preliminary calculations using cut-off 0.10 (OD_{bov}) as defined in this study indicates a test specificity of 94% ($^{48}/_{51} \times 100$).

Rhinoceroses 30, 33 and 48 originated from BTB-free areas but reacted moderately to stimulation by bovine PPD compared with avian PPD stimulation. This can be regarded as non-specific reactions related to exposure to closely related mycobacteria ubiquitously present in the environment, or recent exposure to unrelated microorganisms as described in cattle (McDonald et al., 1999). It is evident that BTB is endemic in buffaloes in the KNP (De Vos et al., 2001; Rodwell et al., 2001), and buffaloes have been shown to serve as sources of infection for other species (Munang'andu et al., 2011) such as rhinoceroses that may share water and grazing areas. Irrespective of the BTB status of the resident wildlife populations, none of the animals reacted exceptionally strongly to the mycobacterial antigens when compared to the reactions elicited by the mitogen (Figs 2 and 3). We therefore conclude that these findings support the absence of BTB infection in all 74 rhinoceroses, including the 23 animals from the KNP.

To eliminate or reduce potential cross-reactivity, it may be useful to include the use of fusion protein ESAT-6/CFP10, (Aagaard et al., 2006), which is a potent T-cell antigen that is used in cattle for differentiating between non-vaccinated and BCG-vaccinated cattle (Buddle et al., 1999; Vordermeier et al., 2002). Alternatively, antigen peptide cocktails as used in IFN-γ assays in other species may be employed (Vordermeier et al., 2001; Casal et al., 2012). The ESAT-6 gene is present in *M. tuberculosis* and virulent *M. bovis* strains but absent from *M. bovis* BCG and environmental acid-fast bacteria (Harboe et al., 1996).

Validation of diagnostic tests in wildlife species is extremely difficult due to unavailability or inaccessibility of samples. For diseases such as BTB, stringent criteria are required by OIE standards for test validation (OIE Terrestrial Manual, 2012). These criteria are directed at domesticated species and thus difficult to achieve for diagnostic tests developed for valuable wildlife species such as

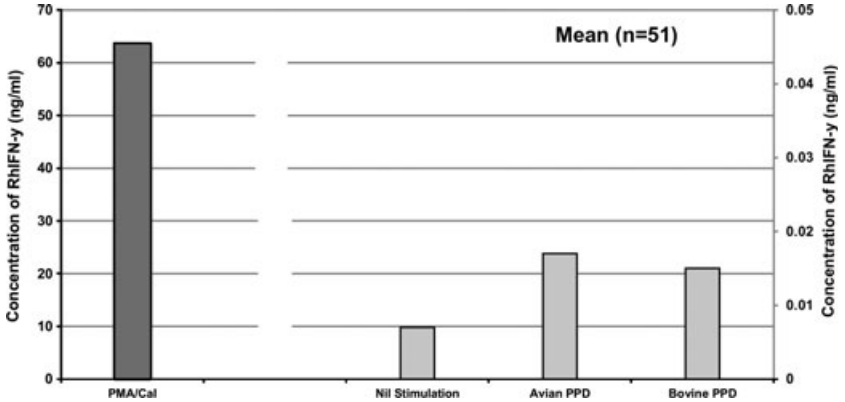

Fig. 3. Averaged IFN-γ responses from whole-blood samples of 51 rhinoceroses from BTB-free areas only, expressed in ng/ml, after 48 h stimulation. Conditions indicated are not corrected for the nil value.

rhinoceroses; therefore, guidelines for validation of diagnostic tests for use in wildlife species are needed.

In this study, we were able to sample 52 rhinoceroses from known BTB-free areas and 23 from the BTB-endemic KNP. Given the target population in this study, selection of an appropriate cut-off value for a negative test result was used to determine the test performance of the assay. The proposed cut-off value identified three animals in the BTB-free areas ($n = 51$, OD_{bovine} $\mu = 0.02$, $\sigma = 0.04$) as false-positive reactors.

At this stage, the chosen cut-off value for a negative test result is a preliminary recommendation based on observed IFN-γ responses in BTB-free rhinoceroses only and may in all likelihood change, possibly in favour of improved specificity, during the characterization of the diagnostic sensitivity of this assay.

The rhinoceros-specific IFN-γ assay detects native rhinoceros IFN-γ from supernatant harvested from whole-blood cultures stimulated with mitogens. The results from the present study indicate that the ELISA is capable of correctly classifying BTB-negative rhinoceroses. The initial results obtained show its promise as a diagnostic tool for BTB infections in captive as well as free-ranging rhinoceroses, but it will require further validation with blood samples of animals known to be BTB-infected.

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Conflicts of Interest

None of the authors have any conflicts of interest.

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