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Research paper

An interferon-gamma release assay for the diagnosis of the *Mycobacterium bovis* infection in white rhinoceros (*Ceratotherium simum*)

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ABSTRACT

Mycobacterium bovis (*M. bovis*), the cause of bovine tuberculosis, is endemic in Kruger National Park (KNP), South Africa. The risk of spread of *M. bovis* infection currently prevents translocation of white rhinoceros (*Ceratotherium simum*) from this population. Therefore, accurate assays are necessary for screening this threatened species. Interferon gamma (IFN-γ) release assays (IGRA) are commonly used for tuberculosis diagnosis in humans and other wildlife species. Hence, the aim of this study was to develop an IGRA for *M. bovis* detection in white rhinoceros. Heparinized whole blood was collected from immobilized white rhinoceros in KNP (n = 131) and incubated overnight in QuantiFERON®-TB Gold (QFT) blood collection tubes, after which the plasma was harvested following centrifugation. Tissue samples for mycobacterial culture were available from a subset of 21 rhinoceros. The concentration of IFN-γ in plasma samples was measured using the Mabtech equine IFN-γ ELISA^{PRO} kit. An IGRA result was calculated as the difference in IFN-γ concentrations in the QFT Nil and TB antigen tubes. Using test results for the white rhinoceros with known infection status, a diagnostic cut-off value was calculated as 21 pg/ml. Additionally, cut-off values for IFN-γ concentrations for plasma from QFT Nil and QFT Mitogen tubes were calculated to increase confidence in IGRA result interpretation. The combination of the QFT stimulation platform and Mabtech equine IFN-γ ELISA is a promising diagnostic test to distinguish between of *M. bovis*-infected and -uninfected white rhinoceros.

1. Introduction

The white rhinoceros (*Ceratotherium simum*) is threatened (Emslie, 2011), and under severe poaching pressure. The largest populations of white rhinoceros are found in Kruger National Park (KNP), South Africa, where bovine tuberculosis (bTB) is endemic (Renwick et al., 2007). Bovine tuberculosis is a chronic disease caused by infection with *Mycobacterium bovis* (*M. bovis*), a member of the pathogenic *M. tuberculosis* complex (MTBC) (Brosch et al., 2002). Although white rhinoceros are susceptible to infection with *M. bovis*, there remains a knowledge gap concerning the extent of infection in exposed populations (Miller et al., 2018). In order to improve our understanding of bTB in white rhinoceros and prevent the spread of the disease through translocation of infected animals, new diagnostic tools are required to accurately identify infected and possibly diseased individuals.

Infection with *M. bovis* is usually diagnosed by detecting cell-mediated immune (CMI) responses towards pathogen-specific antigens (Welsh et al., 2005). This can be achieved by using an interferon-gamma (IFN-γ) release assay (IGRA) which includes antigenic stimulation of blood lymphocytes followed by measurement of the release of IFN-γ using an enzyme-linked immunosorbent assay (ELISA). Interferon-gamma is a commonly used cytokine biomarker for detection of MTBC infection and diagnostic IGRAs for detection of *M. bovis* infection have been developed for cattle (Bernitz et al., 2018), buffaloes (Goosen et al., 2014), and wild dogs (Higgitt et al., 2017).

The QuantiFERON-TB Gold (In-Tube) (QFT) system was developed for a human IGRA which provides within sterile blood collection tubes a peptide cocktail simulating the antigens; early secreted antigenic target

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6 kDa protein (ESAT-6), culture filtrate protein 10 (CFP-10) and TB7.7 (Ruhwald et al., 2007). Use of the QFT blood collection tubes has been advocated for field testing of African wildlife because they are easy to use, provide high diagnostic specificity (Parsons et al., 2011), and can be adapted for use in multiple species (Bernitz et al., 2018; Higgitt et al., 2017; Roos et al., 2018). In addition, the QFT system has been previously used by Parsons et al. (2017) to measure immune sensitization of white rhinoceros to *M. bovis*. Therefore, the aim of this study was to describe a diagnostic QFT IGRA for detection of *M. bovis* infection in white rhinoceros by combining the commercially available QFT stimulation platform with a validated rhinoceros IFN-γ ELISA (Chileshe et al., 2019). This validated IFN-γ ELISA was selected based on phylogenetic relationship between rhinoceros (Steiner and Ryder, 2011) and equine species and the high homology of the IFN-γ sequences (Morar et al., 2007).

2. Materials and methods

2.1. Animals and samples

Whole blood was collected opportunistically in heparinized tubes (BD Biosciences, Franklin Lakes, New Jersey, USA) from 131 chemically immobilized white rhinoceros in KNP between 2015 and 2018, as previously described (Miller et al., 2018), during routine management practices or for other approved activities. Twenty-one of these animals were subsequently euthanized due to severe poaching wounds. From these 21 rhinoceros, post-mortem tissue samples were collected (specifically, submandibular, retropharyngeal, cervical, prescapular, axillary, inguineal, mediastinal, tracheobronchial, mesenteric lymph nodes and lung) and frozen for transport to the lab, as previously described (Miller et al., 2018). All samples were processed for mycobacterial culture using the BACTEC™ MGIT™ 960 system (BD Biosciences) as described by Goosen et al. (2014). All Ziehl-Neelsen stain-positive bacterial cultures were speciated using genetic region of difference analysis (Warren et al., 2006). Results were used to define animals as *M. bovis*-infected if any tissue was culture-positive, or uninfected if all tissues were culture-negative.

Also included in this study, 10 archived plasma sample sets obtained from two white rhinoceros that had been experimentally infected with *M. bovis* and tested monthly using the QFT system, as previously described (Parsons et al., 2017). These two white rhinoceros were included in the *M. bovis*-infected cohort for ROC curve analyses.

2.2. Interferon gamma (IFN-γ) release assay (IGRA)

One ml aliquots of heparinized whole blood were incubated in tubes of the QFT system (Qiagen, Venlo, Limburg, Netherlands) comprising a Nil tube (containing saline) and a TB Antigen (TB Ag) tube containing mycobacterial peptides (ESAT6, CFP10, TB7.7). Additionally, one ml aliquots were incubated in either QFT Mitogen tubes (n = 90) or in tubes containing pokeweed mitogen (Sigma-Aldrich, St. Louis, Missouri, USA) at a final concentration of 10 μg/ml (n = 41). All tubes were incubated for 24 h at 37 °C. After incubation, plasma was harvested following centrifugation at 800 × *g* for 10 min, transferred into a 2 ml microcentrifuge tube, and frozen immediately at −80 °C until analysis, usually less than one month.

All plasma samples, including samples from the experimentally infected animals, were analyzed using the equine IFN-γ ELISA^{PRO} kit (Mabtech Ab, Nacka Strand, Sweden; custom precoated plates using kit reagent product 3117-1H-6) as previously described (Chileshe et al., 2019). The IFN-γ concentrations in plasma from the Nil, TB Ag and mitogen tubes were defined as [IFN-γ^{Nil}], [IFN-γ^{TB}], [IFN-γ^{Mit}], respectively, and the QFT interferon gamma release assay (IGRA) result for each rhinoceros was calculated as [IFN-γ^{TB}]-[IFN-γ^{Nil}], measured in pg/ml.

2.3. Data analysis

The QFT test values of the culture-confirmed naturally *M. bovis*-infected and –uninfected rhinoceros (n = 21) were compared using the unpaired Student *t*-test. A p value of 0.05 was used to determine statistical significance.

A receiver operator characteristic (ROC) curve analysis was performed to determine the optimal diagnostic cut-off value for the QFT assay, calculated as the value that yielded the highest Youden's index (Youden, 1950). Hereafter, alternative cut-off values for the QFT assay were evaluated in the remaining 110 free-ranging rhinoceros (without culture results) by comparing the proportion of animals testing positive for each alternative value to the proportion testing positive using the ROC derived cut-off. This was done using a Z test (GraphPad Software, version 5, Inc., La Jolla, CA, USA). For this analysis, a stringent p value of ≤ 0.01 was considered statistically significant. In order to ensure the validity of future tests, exclusion criteria for the QFT Nil and QFT Mitogen samples were calculated as the 95th percentile of all [IFN-γ^{Nil}] values (n = 131) and the 5th percentile of all [IFN-γ^{Mit}] values (n = 90), respectively. The cut-off values were used to determine thresholds for negative (QFT Nil) and positive (QFT Mitogen) control samples.

3. Results

Of the 21 white rhinoceros that had mycobacterial culture results, 8 were *M. bovis* culture-positive and 13 were negative. The *M. bovis*-infected rhinoceros had localized disease, with small culture-positive lesions found in the head and/or thoracic lymph nodes and intermittently in the lungs. The 21 test results for these animals are shown in Fig. 1. The mean QFT IGRA value for *M. bovis*-infected animals (n = 8) was significantly greater than the mean value for uninfected animals (Fig. 1; p < 0.001). Based on ROC curve analysis, the optimal QFT IGRA cut-off value was 21 pg/ml (Se = 78%, 95% CI 52–93%; Sp = 92%, 95% CI 63–99%; AUC = 0.84) (Fig. 2, Table 1).

Using this cut-off value, 21 out of 110 (19%) white rhinoceros (with no culture results) were test positive. The distribution of QFT IGRA responses for *M. bovis*-exposed rhinoceros is shown in Fig. 3. The proportion of animals testing positive using a cut-off value of 21 pg/ml was compared with the proportion of animals testing positive using proposed positive cut-off values (as listed in column 1, Table 1). Cut-off values lower than 21 pg/ml and greater than 61 pg/ml resulted in significantly different proportions of test-positive rhinoceros.

In order to determine exclusion criteria for the QFT IGRA, the 95th percentile of all [IFN-γ^{Nil}] values was calculated as 28 pg/ml and the

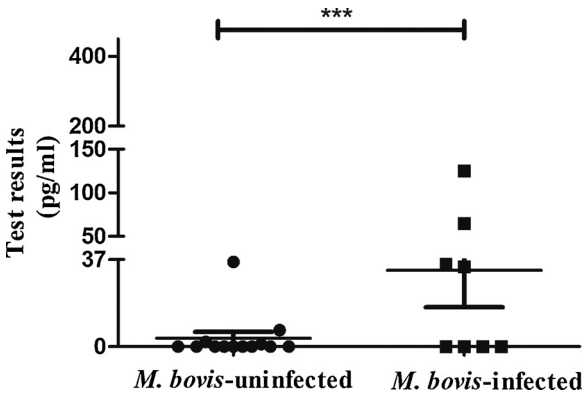

Fig. 1. The QuantiFERON-TB Gold In-Tube interferon gamma release assay results for *M. bovis*-infected and uninfected white rhinoceros, showing a significant difference between the means of these groups (unpaired Student *t*-test *** p < 0.001). Means and 95% confidence intervals are shown by horizontal bars.

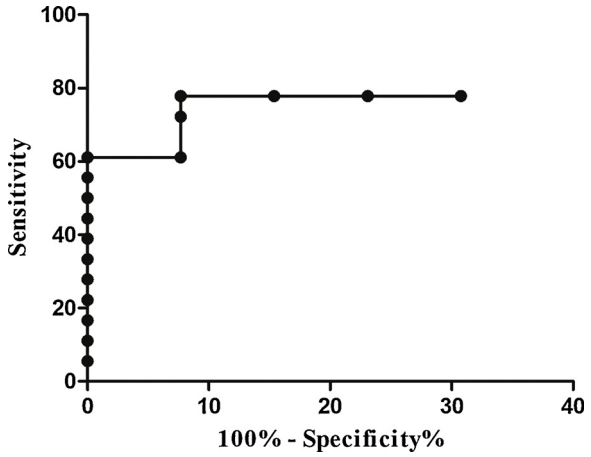

Fig. 2. Receiver operating characteristics curve analysis of QuantiFERON-TB Gold In-Tube interferon gamma release assay results for *Mycobacterium bovis*-infected and uninfected white rhinoceros (Area Under the Curve = 0.84; 95% CI, 0.70-0.98; $p < 0.001$).

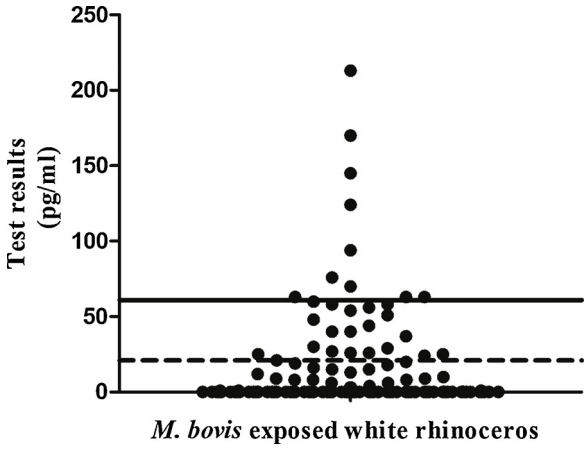

Fig. 3. QuantiFERON-TB Gold In-Tube interferon gamma release assay values of 110 *M. bovis*-exposed white rhinoceros from Kruger National Park. The optimal cut-off value of 21 pg/ml is indicated by the dotted line (————) and the proposed positive cut-off value of 61 pg/ml indicated by straight line (——).

5th percentile of [IFN-γ^{Mit}] values as 84 pg/ml (Table 2).

4. Discussion

In this study, a new diagnostic IGRA, combining the QFT stimulation platform and Mabtech equine IFN-γ ELISA^{PRO} kit, was evaluated for detection of *M. bovis* infection in white rhinoceros. The IGRA results for *M. bovis*-infected animals were significantly greater than those for uninfected animals, indicating that it could be used to distinguish between these groups. The optimal cut-off value was calculated as 21 pg/ml of antigen-specific IFN-γ. In addition, we calculated cut-off values for IFN-γ concentrations for plasma from QFT Nil and QFT Mitogen tubes to increase confidence in IGRA result interpretation.

White rhinoceros with confirmed *M. bovis* infection status were used to determine the diagnostic cut-off value. The value of 21 pg/ml is similar to cut-off values determined for QFT IGRAs in wild dogs (51 pg/ml) (Higgitt et al., 2019), African buffaloes (66 pg/ml) (Parsons et al., 2011) and humans (18 pg/ml) (Ruhwald et al., 2007). Therefore, this diagnostic cut-off value appears appropriate for white rhinoceros. However, the low number of animals used for this analysis, and the wide confidence intervals obtained for test parameters, suggests that this value should be considered as a preliminary finding. However, in a larger cohort, QFT test outcomes using a cut-off of 21 pg/ml were statistically similar to those using cut-off values ranging from 35 to 61 pg/ml. For these reasons, we propose that QFT IGRA results be interpreted as follows: a test result < 21 pg/ml indicates that the rhinoceros is uninfected; a test result > 61 pg/ml, that the rhinoceros has detectable immune sensitization to *M. bovis* antigens, consistent with infection; and results ranging from 21 to 61 pg/ml should be considered suspect for infection. Interpretation of the latter should be informed by epidemiological data and risk management considerations. Therefore, serial testing is recommended since a single result only provides an insight into the current status and not progression of initial infection.

Table 2
Interpretation criteria for the QuantiFERON-TB Gold In-Tube interferon gamma release assay.

Test result	[IFN-γ^{Nil}]		[IFN-γ^{Mit}]	[IFN-γ^{TB}] minus [IFN-γ^{Nil}]
Negative	≤ 28 pg/ml	AND	≥ 84 pg/ml	≤ 21 pg/ml
Positive	≤ 28 pg/ml	AND	≥ 84 pg/ml	≥ 21 pg/ml
Inconclusive	≥ 28 pg/ml	OR	≤ 84 pg/ml	N/A

IFN-γ, interferon gamma; [IFN-γ^{Nil}] concentration in nil tube; [IFN-γ^{Mit}], concentration in Mitogen tube; [IFN-γ^{TB}], concentration in TB antigen tube; N/A, not applicable.

Table 1
Test performance of the QuantiFERON-TB Gold In-Tube interferon gamma release assay, for selected cut-off values as determined by receiver operating characteristic curve analysis using samples (n = 31) from *M. bovis*-infected and uninfected white rhinoceros.

Cut-off value (pg/ml)	Sensitivity %	95% CI	Specificity %	95 % CI	Youden's Index %
1	78	52.3% – 93.5%	69	38.5% to 90.9%	47
2	78	52.3% – 93.5%	77	46.1% to 94.9%	55
5	78	52.3% – 93.5%	85	54.5% to 98.1%	62
[a]21	78	52.3% – 93.5%	92	63.9% to 99.8%	70
35	72	46.5% – 90.3%	92	63.9% to 99.8%	65
36	61	35.7% – 82.7%	92	63.9% to 99.8%	53
41	61	35.7% – 82.7%	100	75.3% to 100%	61
48	56	30.7% – 78.4%	100	75.3% to 100%	56
54	50	26.0% – 73.9%	100	75.3% to 100%	50
61	44	21.5% – 69.2%	100	75.3% to 100%	44
69	39	17.3% – 64.2%	100	75.3% to 100%	39
77	33	13.3% – 59.0%	100	75.3% to 100%	33
85	28	9.6% – 53.4%	100	75.3% to 100%	28
99	22	6.4% – 47.6%	100	75.3% to 100%	22
112	17	3.5% – 41.4%	100	75.3% to 100%	17
121	11	1.3% – 34.7%	100	75.3% to 100%	11
272	6	0.1% – 27.2%	100	75.3% to 100%	6

[a] Optimal diagnostic cut-off value; CI, confidence interval.

In order to confirm the validity of test results, cut-off values for the QFT Nil and QFT Mitogen samples were calculated as < 28 pg/ml and > 84 pg/ml, respectively. The latter value is stringent when compared to the human QFT and Qiagen Cattletype® IGRAs that use cut-off values for mitogen samples that are similar to the diagnostic cut-off value (Ruhwald et al., 2007; Bernitz et al., 2018). The interpretation criterion for the mitogen sample is important for confirming viability and function of cells throughout the stimulation process. Individual rhinoceros samples with QFT Mitogen values that do not have an IFN-γ concentration ≥84 pg/ml should be excluded since a negative QFT IGRA value cannot be reliably interpreted as negative. With regards to background IFN-γ concentration in the QFT Nil sample, high non-specific values may occur if there is a technical issue in blood processing or if the individual has other conditions causing in vivo immune stimulation (Pai et al., 2014). Therefore, these cut-off values provide increased confidence in test interpretation.

The QFT IGRA has shown utility for screening white rhinoceros to detect *M. bovis* infection. In addition, this IGRA is easy to use and incorporates commercially available reagents to improve reproducibility. Notably, the use of the prepared tubes of the QFT stimulation platform is especially suited for field use.

Limitations of the study included the low number of culture-confirmed white rhinoceros in the *M. bovis*-infected and uninfected groups. Also, the true infection status of the larger cohort of 110 white rhinoceros was unconfirmed. This highlights the difficulty of validating diagnostic tests in threatened species with few individuals with confirmed infection. In addition, a test result < 21 pg/ml indicates that the rhinoceros is uninfected, although it could be an early infection that has not yet resulted in a detectable immune response. With a test result > 61 pg/ml, the rhinoceros has detectable immune sensitization to *M. bovis* antigens, consistent with infection, although it could also have recently cleared infection.

In conclusion, this study shows the combination of the QFT stimulation platform and Mabtech equine IFN-γ ELISA^{PRO} is a promising diagnostic test for the detection of *M. bovis* infection in white rhinoceros. However, further research is necessary to recalculate the cut-off value of this assay using a ROC curve analysis with samples from larger infected and unexposed populations, respectively.

Declaration of Competing Interest

The authors report no conflict of interest.

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