



Research paper

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



Josephine Chileshe^a, Eduard O. Roos^{a,1}, Wynand J. Goosen^a, Peter Buss^b, Guy Hausler^b, Leana Rossouw^b, Tebogo Manemela^b, Paul van Helden^a, Robin Warren^a, Sven DC Parsons^a, Michele Miller^{a,*}

^a Department of Science and Technology-National Research Foundation Centre of Excellence for Biomedical Tuberculosis Research, South African Medical Research Council Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, PO Box 241, Cape Town, 8000, South Africa

^b Veterinary Wildlife Services, South African National Parks, Kruger National Park, Private Bag X402, Skukuza, 1350, South Africa

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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

* Corresponding author.

E-mail address: miller@sun.ac.za (M. Miller).

¹ Present address: The Pirbright Institute, Ash Rd, Pirbright, Woking GU24 0NF, United Kingdom.

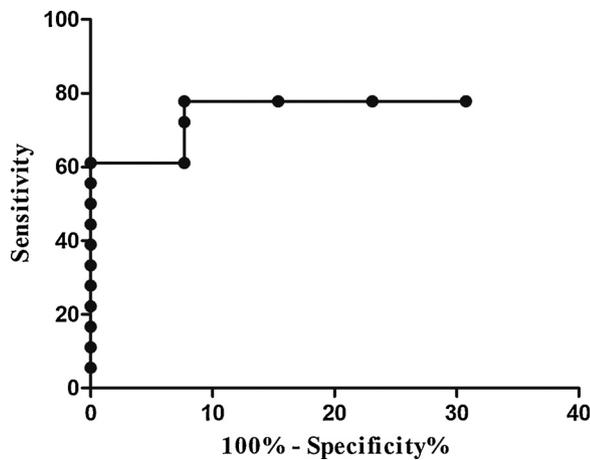


Fig. 2. Receiver operating characteristics curve analysis of QuantiFERON-TB Gold In-Tube interferon gamma release assay results for *Mycobacterium bovis*-infected and uninfected white rhinoceros (Area Under the Curve = 0.84; 95% CI, 0.70-0.98; $p < 0.001$).

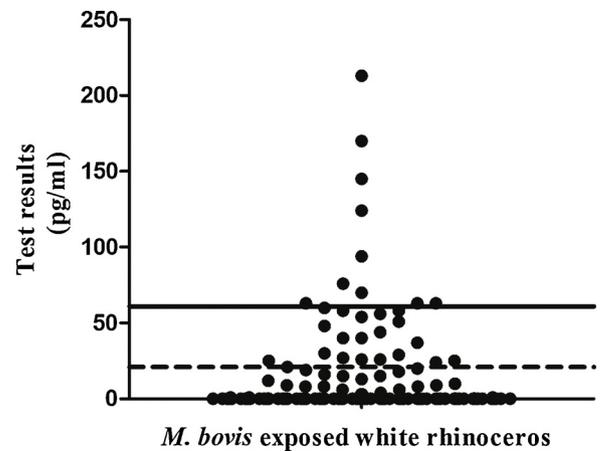


Fig. 3. QuantiFERON-TB Gold In-Tube interferon gamma release assay values of 110 *M. bovis*-exposed white rhinoceros from Kruger National Park. The optimal cut-off value of 21 pg/ml is indicated by the dotted line (-----) and the proposed positive cut-off value of 61 pg/ml indicated by straight line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

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

4. Discussion

In this study, a new diagnostic IGRA, combining the QFT stimulation platform and Mabtech equine $IFN-\gamma$ 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 $IFN-\gamma$. In addition, we calculated cut-off values for $IFN-\gamma$ 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

Table 2

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

Test result	$[IFN-\gamma^{Nil}]$		$[IFN-\gamma^{Mit}]$	$[IFN-\gamma^{TB}]$ minus $[IFN-\gamma^{Nil}]$
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

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

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 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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