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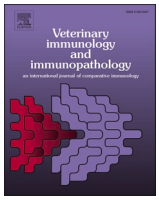

Short communication

Reduced capability of refrigerated white rhinoceros whole blood to produce interferon-gamma upon mitogen stimulation

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ABSTRACT

Ante-mortem surveillance for *Mycobacterium bovis* (*M. bovis*) infection in the Kruger National Park (KNP) rhinoceros population currently relies on results from the QuantiFERON-TB Gold (In-Tube) Plus (QFT)–interferon gamma (IFN-γ) release assay (IGRA). However, same-day processing of rhinoceros blood samples for this test is a logistical challenge. Therefore, a pilot study was performed to compare mitogen-stimulated and unstimulated IFN-γ concentrations in plasma from rhinoceros whole blood processed within 6 h of collection or stored at 4°C for 24 and 48 h prior to incubation in QFT tubes. Replicate samples of heparinized whole blood from seven subadult male white rhinoceros were used. Results showed no change in IFN-γ levels in unstimulated samples, however the relative concentrations of IFN-γ (based on optical density values) in mitogen plasma decreased significantly with increased time blood was stored post-collection and prior to QFT stimulation. These findings support a need for same-day processing of rhinoceros blood samples for QFT-IGRA testing as per the current practice. Further investigation using TB-antigen stimulated samples is warranted to properly assess the impact of blood storage on TB test results in rhinoceros.

1. Introduction

The survival of white rhinoceros (*Ceratotherium simum*) populations is threatened by poaching, habitat loss, drought, and potentially infectious diseases, such as bovine tuberculosis (Miller et al., 2018; Dwyer et al., 2020). *Mycobacterium bovis* infection, which is the primary cause of bovine tuberculosis (TB), is present in African rhinoceros in Kruger National Park (KNP), South Africa (Miller et al., 2018; Goosen et al., 2022). Ante-mortem surveillance of *M. bovis* infection in this population currently relies on an in vitro cytokine release assay called the QuantiFERON-TB Gold (In-Tube) Plus (QFT)–interferon gamma release assay (IGRA), which has been recently validated for white rhinoceros (Chileshe et al., 2019a, 2019b). This process requires that heparinized whole blood samples collected from immobilized rhinoceros are transported to laboratories and processed the same day to perform whole blood incubation in QFT tubes.

This requirement is a logistical challenge for using the QFT-IGRA for TB detection. Rhinoceros in national parks and game reserves are often located in remote areas, without easy access to laboratory facilities or sample transport options, or with inconsistent availability of personnel to process these samples timeously upon their arrival at laboratories. Therefore, it is important to investigate the impact of extended whole blood storage (at 4 °C to mimic transport conditions/extended delays) on the ability of immune cells to produce interferon-γ (IFN-γ) when stimulated in vitro. The goal of this pilot study was to measure and compare mitogen-stimulated IFN-γ production in rhinoceros whole blood processed within 6 h of collection, to that in replicate samples stored at 4°C for 24 and 48 h prior to stimulation. These results will inform whether transport/processing delays could impact QFT-IGRA results in rhinoceros.

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2. Materials and methods

2.1. Animals

Whole blood samples were opportunistically collected from the metacarpal vein of seven clinically normal subadult male white rhinoceros that were chemically immobilised by experienced wildlife veterinarians in Kruger National Park (KNP), South Africa, for dehorning procedures. Immobilisations were performed according to the Standard Operating Procedures for the Capture, Transportation and Maintenance in Holding Facilities of Wildlife (South African National Parks). Ethical approval for this project was granted by the Stellenbosch Animal Care and Use Committee (SU-2020–19019), and a section 20 research permit was issued by the Department of Agriculture, Land Reform and Rural Development (DALRRD;12/11/1/7/2).

2.2. Whole blood stimulation

Rhinoceros whole blood was collected in 9 mL lithium heparin vacutainers (BD Biosciences, Franklin Lakes, New Jersey, USA), as described by Chileshe et al. (2019a, 2019b). Samples were transported at ambient temperature (approximately 18–23 °C) in a Styrofoam container to the Veterinary Wildlife Services laboratory within 6 h of collection. A 2 mL aliquot of blood was removed (t = 0 h) after arrival at the laboratory and 1 mL added to each of the QFT (Qiagen, Venlo, Limburg, The Netherlands) nil (containing saline), and mitogen (containing phytohaemagglutinin) tubes. To ensure sufficient stimulation, additional pokeweed mitogen (Sigma Aldrich, St. Louis, Missouri, USA) was added to the QFT mitogen tube (10 μL) at a final concentration of 10 μg/mL. Tubes were thoroughly inverted 10 times then transferred to a 37 °C incubator for 24 h. The remaining heparinized whole blood was refrigerated for 24 h at 4 °C, then allowed to warm to room temperature, prior to adding aliquots to a set of QFT tubes, as described above. For the three rhinoceros with sufficient remaining volume of heparinized whole blood, an additional aliquot was stored at 4 °C for 48 h prior to adding the sample to a set of QFT tubes. All QFT tube sets were incubated at 37 °C for 24 h. After incubation, plasma was harvested following centrifugation at 800 x *g* for 10 min, transferred to a 2 mL microcentrifuge tube, and frozen immediately at − 80 °C until testing (completed within 1 month).

2.3. Interferon-gamma (IFN-γ) ELISA

Interferon gamma detection in the nil and mitogen plasma samples was performed using the anti-equine IFN-γ ELISA^{PRO} kit (Mabtech Ab, Nacka Strand, Sweden; custom precoated plate using reagent product 3117 – 1 H – 6). The procedure, as previously described (Chileshe et al., 2019a, 2019b), was conducted with slight modifications; all mitogen plasma samples were serially diluted 1:10, 1:100, 1:1000 and 1:10,000, and nil plasma samples were diluted 1:2, in ELISA sample diluent prior to adding each sample to duplicate wells. The remainder of the assay steps were followed as previously reported (Chileshe et al., 2019b).

2.4. Data analysis

The mean optical density (OD) values for each sample were calculated after subtracting OD_{630} value from OD_{450} value for each well. The mean OD values of the serial dilutions for each rhinoceros was then compared to the working range of OD values of the standard curve in order to select the appropriate dilution for comparison of IFN-γ concentrations in the replicate samples at each time point. This step was performed to use empirical OD values for comparison rather than extrapolating IFN-γ concentrations in the mitogen samples, which were expected to be outside the linear range of the assay. A linear mixed effects model (fit with R statistical software (R Core Team, 2020) using packages lme4 (Bates et al., 2015) and lmertest (Kuznetsova et al., 2017) was then used to compare mean OD values in mitogen plasma from whole blood stored at 4 °C for 0, 24, and 48 h. The overall model outcome was the mean OD value at the selected dilution, and the main fixed effect was storage time. Random effects for intercept were included for each animal to account for repeated measures on the same individual. Likelihood ratio tests compared models with and without the fixed effect of storage time to generate a p-value. A p-value < 0.05 was considered statistically significant.

3. Results and discussion

The mean OD results for unstimulated (nil) rhinoceros samples, diluted 1:2, were all near zero (OD < 0.01), which was below the assay's limit of quantification based on IFN-γ concentration (LOQ = 7.8 pg/mL; Chileshe et al., 2019b). These results were similar at all three storage time points for all individual rhinoceros. Since these results indicated that there was no production of IFN-γ in the unstimulated rhinoceros samples, no further analyses were undertaken. Therefore, refrigerated storage of rhinoceros whole blood for up to 48 h did not appear to affect background levels of IFN-γ.

The mitogen sample mean OD values were all significantly higher than mean OD values for the corresponding nil samples (Supplementary Figure 1), which suggested stimulation of IFN-γ production in blood processed after all storage time points. To compare empirical measurements of IFN-γ production in mitogen samples across time points, the dilution factor resulting in an OD value in the range of 0.07–1.00 was selected for each set of samples from an individual rhinoceros. The dilution factor chosen was 1:100 plasma dilution for two rhinoceros and 1:1000 plasma dilution for five rhinoceros. Since the dilution factor was kept constant for a given individual rhinoceros, selecting different dilution factors for different rhinoceros did not affect the comparisons. Mean OD values for each set of mitogen samples from an individual rhinoceros decreased as whole blood storage time increased (Fig. 1). The results from a linear mixed model, that included storage time as the fixed effect and individual rhinoceros as a random effect, are shown in Table 1. These findings confirmed that there was a significant decrease in mean mitogen sample OD values when whole blood was stored at 4 °C for 24 and 48 h prior to stimulation (−2 log likelihood of the reduced model containing the intercept only compared to the full model with time as a fixed effect = 13.63; $\chi^2 = 12.75$, p = 0.002). Therefore, these pilot data suggest that storage of rhinoceros whole blood at 4 °C for 24–48 h may impact the ability of immune cells to produce IFN-γ when stimulated in vitro.

A diagnostic cut-off value for mitogen induced interferon gamma responses in rhinoceros has been previously determined (Chileshe et al., 2019a, 2019b) to be > 84 pg/mL, although IFN-γ concentrations are typically greater than 400 pg/mL. All specimens in this study met this criterion at each time point, indicating that processed blood retained a sufficient, albeit reduced, ability to produce IFN-γ upon mitogen stimulation. Antigen-specific IFN-γ concentrations in *M. bovis* sensitised rhinoceros have ranged between 22 and 700 pg/mL (Chileshe et al., 2019b; Dwyer et al., 2022), and were usually lower than Mit-stimulated IFN-γ concentrations. One of the limitations of this study was the inability to select known *M. bovis* sensitized rhinoceros at the time of sample collection since the animals were free ranging. Therefore, data are unavailable to determine whether delays in processing decreases IFN-γ production enough to change the test positive to negative classification; this would require further investigation to confirm.

The rationale for this pilot study was based on the limited resources available to veterinary staff collecting and transporting samples in the context of Kruger National Park and other areas in South Africa, as well as incorporating the manufacturer's guidelines for use of the QFT system with human blood (Qiagen, 2019) for IGRA. These state that human blood samples should be kept at 17–25 °C, for a maximum of 12 h, or transferred to storage at 2–8 °C within 3 h of collection for a maximum of 48 h prior to processing. However, limited studies conducted in

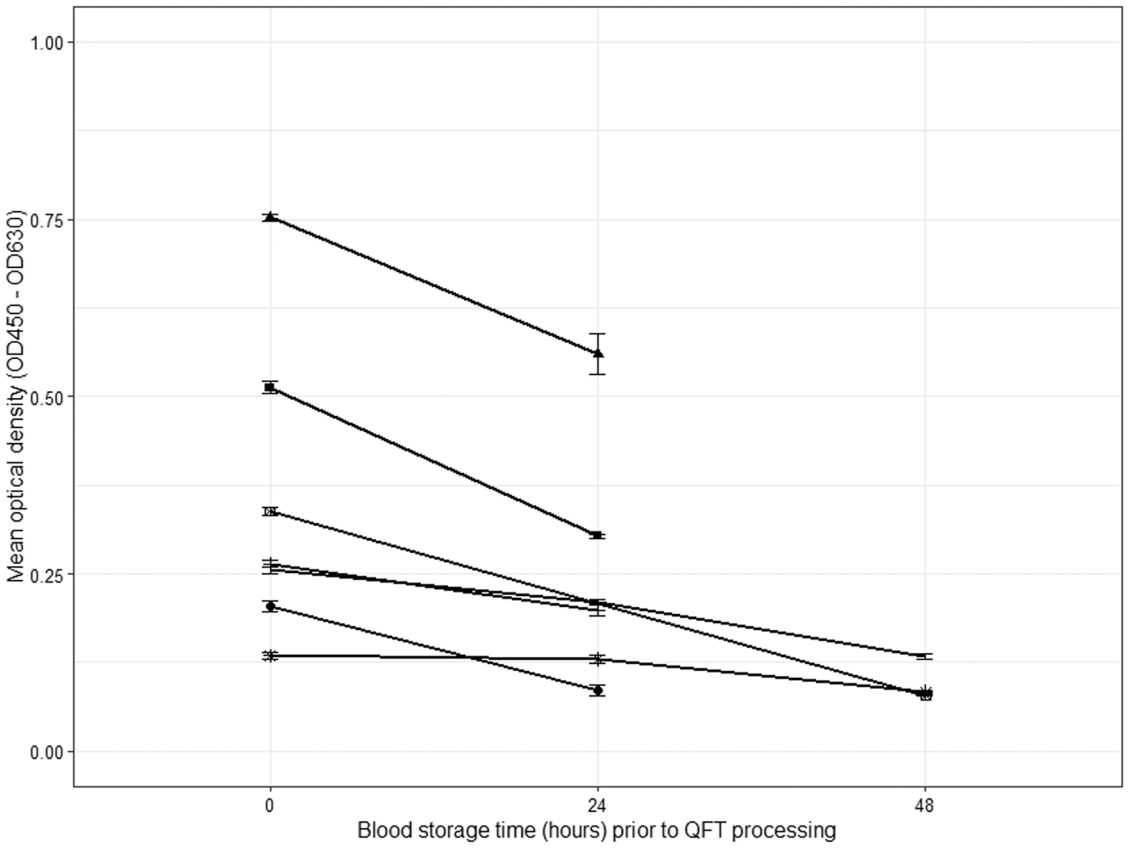

Fig. 1. Mean optical density (OD) results for plasma harvested from QuantiFERON-TB Gold (In-Tube) Plus (QFT) mitogen tubes and measured in the equine interferon-gamma (IFN-γ) ELISA are shown. A fixed plasma dilution factor for each of seven white rhinoceros (represented by different symbols) was selected and OD values shown for each whole blood storage time point (0, 24, 48 h at 4 °C prior to stimulation). Error bars indicate standard deviation across two replicates for each mean OD result. Note: for one individual, there was insufficient plasma volume to complete the IGRA on the Nil and Mit samples from the 24 h test point; hence, only the 0 and 48 h timepoint samples could be tested for this animal.

Table 1
Linear mixed model of the relationship between mean optical density (OD) values of plasma from QuantiFERON-TB Gold (In-Tube) Plus (QFT) mitogen-stimulated rhinoceros whole blood screened with an equine interferon-gamma (IFN-γ) ELISA, and the time blood was stored at 4 ºC prior to stimulation (n = 17 observations). Random effects for intercept were included for individual animals.

	Coefficient	Standard error	df[$]	p[%]	-2 log likelihood[*]
Intercept	0.35	0.065	8		7.251
24 h storage vs. 0 h	-0.11	0.029	9	0.004	
48 h of storage vs. 0 h	-0.17	0.039	9	0.002	

[$] df – degrees of freedom;
[%] p – p-value for fixed effect;
[*] Storage time was a significant indicator of mean OD value in the uniformly diluted mitogen plasma samples, as determined by comparing the − 2 log likelihood statistic from this model to that of a reduced model containing the intercept and random effects only (−2 log likelihood of the reduced model = 13.63; $\chi^2 = 12.75$, p = 0.002).

humans and other animal species have shown that delays in processing of blood samples (kept at ambient temperature) for antigen and mitogen stimulation can result in compromised viability of cells, and therefore, a reduced capacity for cytokine production in the stimulated sample (Doherty et al., 2005; Gormley et al., 2006; Smith et al., 2009). For example, a study conducted on human blood found that the number of spot-forming cells (T-cells) detected using the ELIspot assay was significantly reduced in samples where processing delays of 4 h (at ambient temperature) occurred, compared to immediately processed aliquots (Smith et al., 2009). A similar study showed that 2-hour delays in processing human blood samples (held at ambient temperature) had a negative impact on the production of IL-4 and IFN-γ following immune stimulation (Doherty et al., 2005).

Various other studies in humans and animals have reported similar negative impacts on the sensitivity of cytokine release assays with delayed sample processing (Gormley et al., 2006). One study showed that delays in sample processing resulted in a significant increase in the expression of several cytokines (e.g., IL-1, IL-6, and IL-8) measured in unstimulated blood (Duvigneau et al., 2003). In this case, both the delay in processing (h) and the temperature at which samples were stored prior to processing (4 ºC or ambient temperature) had an impact on the measured cytokine expression. Interestingly, the effects differed according to the cytokine measured.

Conversely, other studies have shown no impact of delays in sample processing on sensitivity of cytokine release assays (Gormley et al., 2006). Since the effect of storage of blood at different temperatures on cytokine concentration appears to differ according to the host context, a pilot study using rhinoceros whole blood was necessary to determine how sample processing delays would affect IFN-γ production using conditions relevant to the rhinoceros QFT-IGRA. Due to the variability of ambient temperatures under field collections and transport, a single practical temperature condition was used, after receiving samples at the laboratory. In the current study, whole blood samples were refrigerated at 4 ºC for 0–48 h prior to processing in accordance with the QFT manufacturer guidelines for human blood. Specifically, this study was designed to mimic conditions in which blood is collected in the field,

transported to the clinic at ambient temperature within 6 h, and then placed on ice packs for shipment to a laboratory for further processing. In South Africa, many wildlife veterinarians would not have access to equipment required for initial sample processing and therefore, would need to rely on a courier or other service to get the samples to a laboratory.

The comparison of IFN-γ production in mitogen-stimulated rhinoceros whole blood in the present study used mean OD values rather than IFN-γ concentrations extrapolated from the standard curve. This approach was selected because the mitogen samples contained high levels of IFN-γ that were beyond the linear range of the assay, which would lead to the extrapolated concentrations having greater variability and potential inaccuracy. Such variability could bias the results towards not showing a change in assay performance with blood storage time when there truly was one.

To minimize this effect and use empirical values, the mitogen samples were serially diluted. The dilution factor for each rhinoceros, for comparison of replicate samples over storage time points, was selected based on a mean OD value in the working range of the assay. Therefore, empirical changes in OD values (rather than extrapolated concentrations) were compared between time points within an individual rhinoceros sample set, which created a more sensitive approach for detecting change in relative IFN-γ concentrations.

A limitation of this study was that samples were used from rhinoceros with unknown *M. bovis* infection status, and therefore, the impact of blood storage on antigen-specific IFN-γ results could not be evaluated. Since the concentrations of antigen-specific IFN-γ are typically lower than that of the mitogen-stimulated samples (Chileshe et al., 2019b), the decrease associated with blood storage may lead to a false negative result in *M. bovis* sensitised rhinoceros, and reduced assay sensitivity. Therefore, future studies should investigate QFT-IGRA results using stored blood from a cohort of known *M. bovis* sensitized rhinoceros. Based on the preliminary findings of this study, it is recommended that blood samples from white rhinoceros are processed the same day as collection and kept at room temperature prior to stimulations in the QFT platform, to maintain sensitivity of the QFT-IGRA. Delayed processing should be avoided as this could have negative consequences for test interpretation and confidence in reporting results.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetimm.2022.110485.

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