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Research paper

Cytokine biomarker discovery in the white rhinoceros (*Ceratotherium simum*)

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ABSTRACT

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* (*M. bovis*) infection, disrupts conservation programs of threatened species such as the white rhinoceros (*Ceratotherium simum)*. Interferon gamma release assays have been developed for the diagnosis of *M. bovis* infection in rhinoceros, however, the discovery of additional diagnostic biomarkers might improve the accuracy of case detection. The aim of this pilot study was therefore to evaluate a novel unbiased approach to candidate biomarker discovery and preliminary validation. Whole blood samples from twelve white rhinoceros were incubated in Nil and TB antigen tubes of the QuantiFERON® TB Gold (In-Tube) system after which RNA was extracted and reverse transcribed. Using the equine RT^2 profiler PCR array, relative gene expression analysis of samples from two immune sensitized rhinoceros identified *CCL4, CCL8, IL23A, LTA, NODAL, TNF, CSF3, CXCL10* and *GPI* as upregulated in response to antigen stimulation. Novel gene expression assays (GEAs) were designed for selected candidates, i.e. *CCL4*, *CXCL10* and *IFNG*, and analysis of QFT-processed samples showed the *CXCL10* GEA could distinguish between five *M. bovis*-infected and five uninfected rhinoceros. These findings confirm the value of the equine RT^2 profiler PCR array as a useful tool for screening biomarkers for the diagnosis of *M. bovis* infection in rhinoceros.

1. Introduction

Bovine tuberculosis (bTB) caused by *Mycobacterium bovis* (*M. bovis*) is endemic in the Kruger National Park (KNP), South Africa (Miller et al., 2017). The presence of disease in this park has disrupted conservation programs by restricting translocations of wildlife and may also have an impact on the health of infected animals (Emslie, 2011; Miller et al., 2018). The first case of *M. bovis* infection in rhinoceros in KNP was recorded in 2016 (Miller et al., 2017) with additional cases reported subsequently (Miller et al., 2018). The current management plan for bTB screening in rhinoceros in KNP relies on ante-mortem testing to identify infected animals before translocation, thereby reducing the risk of introducing *M. bovis* into new ecosystems (P. Buss pers. comm).

Ante-mortem tests for the diagnosis of bTB are based on detecting cell-mediated immune (CMI) responses. In rhinoceros, the equine interferon gamma (IFN-γ) ELISA^{PRO} kit (Mabtech Ab, Nacka Strand, Sweden) has been validated to measure antigen-specific cytokine release using the QuantiFERON® TB Gold (In-Tube) system (QFT) for stimulation of whole blood (Chileshe et al., 2019). The QFT system uses mycobacterial peptide antigens early secretory antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10) to provide specificity to the IFN-γ release assay (IGRA). However, studies in other species have shown that cytokine biosignatures provide greater diagnostic accuracy than a single analyte (Bernitz et al., 2019; Palmer et al., 2020). Therefore, there is a need to investigate additional biomarkers for detection of *M. bovis* infection in rhinoceros.

Antigen-induced cytokine gene expression, measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR), has been used to identify potential biomarkers for TB diagnosis in humans (Kim et al., 2013) and cattle (Palmer et al., 2020). This approach may be useful for wildlife where there is a lack of species-specific reagents (de Lisle et al., 2002). For example, gene expression assays (GEAs) using candidate biomarkers in the *CXCL* family have shown promise as diagnostic tools in different wildlife species (Olivier et al., 2015; Higgitt

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et al., 2017; Roos et al., 2019). Therefore, the aim of this study was to investigate GEAs as tools to identify potential biomarkers of *M. bovis* infection in white rhinoceros.

2. Materials and methods

2.1. Animals, sample collection and blood stimulation

Samples were collected from 12 white rhinoceros originating from the KNP as previously described (Chileshe et al., 2019). Two animals that had previously been confirmed to be immunologically sensitized to *M. bovis* antigens (R1 and R2), based on the validated interferon-gamma release assay, provided convenient blood samples for initial biomarker discovery. Additionally, blood and tissue samples were opportunistically collected from severely injured animals that were euthanized for welfare reasons (R3 – R12). Mycobacterial cultures, performed on tissue samples as previously described (Miller et al., 2018), were used to classify animals as either *M. bovis*-infected (n = 5) or uninfected (n = 5).

Whole blood was collected in heparinized vacutainer tubes (BD Biosciences, Franklin Lakes, New Jersey, USA) as previously described (Chileshe et al., 2019). Within 6 h of collection, one ml of heparinized blood was added to each tube of the QuantiFERON® TB Gold Plus (In-Tube) system (QFT) (Qiagen, Venlo, the Netherlands), comprising a Nil tube (QFT-Nil, containing saline) and the TB2 antigen tube (QFT-TB, containing ESAT-6 and CFP-10 peptides). Samples from R1 and R3-R12 were incubated for 24 h at 37 °C, while samples from R2 were incubated for 6 h. After incubation, the blood was transferred to a 2 mL microcentrifuge tube and centrifuged at 800 x g for 10 min. Plasma was harvested and the cell pellets resuspended in 1.3 mL RNALater® (Ambion, Austin, Texas, USA) and stored at −80 °C until analyzed.

2.2. RNA extraction

The RNALater®-stabilised samples were centrifuged at 15 000 × g for 2 min and the supernatant discarded before RNA was extracted from the remaining pellet using the RiboPure™ Blood Kit (Ambion), then eluted in a volume of 60 µL, according to the manufacturer's guidelines. Total RNA concentration (ng/µl) and quality (A260/A280 and A260/A230 ratio) were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA).

2.3. Biomarker screening using the equine RT^2 profiler™ PCR array

The QFT-Nil and QFT-TB antigen samples for R1 (24 h) and R2 (6 h) were analysed using the equine RT^2 profiler PCR array (Qiagen). This quantitative real-time (RT-qPCR) array consists of expression assays for 84 cytokine genes, five reference genes, a genomic DNA control, a reverse-transcription control, and a PCR reagents control. The assay was performed according to the manufacturer's instructions. Briefly, 200 ng of RNA from each sample of 60 µL was treated with genomic DNA (gDNA) Wipeout Buffer and reverse-transcribed using the RT^2 First Strand Kit (Qiagen), in a final volume of 20 µL. The PCR reaction mix for each cDNA sample was prepared using the RT^2 SYBR Green qPCR Mastermix (Qiagen) in a final volume of 1300 µL. Ten µl of the reaction mix was dispensed into duplicate wells of a 384-well plate format using an automated electronic pipette (epMotion®, Eppendorf, Hamburg, Germany).

The RT-qPCR was initiated at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min using a 7900HT™ Thermo Cycler (Applied Biosystems, Foster City, California, USA) and threshold cycle (C_T) values for each assay were automatically determined. For each animal, the difference in gene expression in the QFT-TB Antigen sample, relative to the QFT-Nil sample was calculated according to the manufacturer's instructions using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Potential candidate targets were selected for further analysis based on i) intra-assay variability of < 5% and ii) to limit the number of potential biomarkers identified, we chose a fold change cut off value for upregulated cytokines following antigen stimulation of > 2 in either R1 or R2. The RT-qPCR products for R2 were visualized in a 1% agarose gel (Lonza Group, Basel, Switzerland) by electrophoresis.

2.4. Sequencing of selected white rhinoceros gene transcripts

Based on findings from the RT^2 profiler, as described below, candidate biomarkers were selected for further analysis by RT-qPCR, i.e. *CCL4* and *CXCL10*. In addition, *IFNG* was included since interferon-gamma protein (IFN-γ) has been shown to be a biomarker for *M. bovis* infection in rhinoceros (Chileshe et al., 2019). The gene *YWHAZ* was selected as a reference transcript as its expression is stable in whole blood of numerous other species (Olivier et al., 2015; Higgitt et al., 2017; Roos et al., 2019). In order to confirm that published messenger RNA (mRNA) sequences of the white rhinoceros were valid, *CXCL10*, *CCL4*, *IFNG,* and *YWHAZ* sequences of the domestic horse and white rhinoceros were obtained from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov) and aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Using the Primer3Plus online tool (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi), forward and reverse PCR primers were designed to anneal to regions with high homology (Table 1).

Partial or complete coding sequences of these transcripts were amplified from samples of two randomly selected rhinoceros in a total volume of 25 µL consisting of 1 µL of cDNA (reverse transcribed from 200 ng total RNA using QuantiTect Reverse Transcription kit; Qiagen), 12.5 µL of One*Taq*® Hot Start 2x Mastermix with Standard Buffer (New England BioLabs® Inc., Ipswich, Massachusetts, USA), 0.5 µL of each gene-specific forward and reverse primer (final concentration of 0.2 µM; Integrated DNA Technologies, Coralville, Iowa, USA) and 10.5 µL nuclease-free water. The reaction was initiated at 94 °C for 2 min, followed by 45 cycles of 94 °C for 30 s, a PCR-specific annealing temperature (Table 1) for 30 s, and an extension step of 68 °C for 1 min, and concluded with a final extension step at 68 °C for 10 min using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems).

The qPCR products, visualized in a 1% agarose gel (Lonza Group) by electrophoresis, were submitted to the Central Analytical Facility (Stellenbosch University, South Africa) for sequencing using a 3130xl Genetic Analyzer (Applied Biosystems), according to the manufacturer's guidelines, and analysed using Sequencher software version 5.4.6 (Gene Codes, Ann Arbor, Michigan, USA). The resulting white rhinoceros mRNA sequences were submitted to the GenBank® genetic sequence database (http://www.ncbi.nlm.nih.gov/genbank/) (Table 1).

2.5. qPCR design and evaluation of biomarkers using qPCR assays

For the white rhinoceros mRNA sequences, putative exon-exon boundaries and intron sizes were inferred from domestic horse sequences obtained from the Ensembl Genome Browser (http://www.ensembl.org/index.html). Primer3 online software was used to design

qPCR primers, at least one of which spanned an exon–exon boundary (Table 2).

All qPCRs were performed in triplicate using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, California, USA) and consisted of 10 µL iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 1 µL of each gene-specific forward and reverse qPCR primer (at a final primer concentration of 0.5 µM; Integrated DNA Technologies), 2 µL of cDNA, and 6 µL of nuclease-free water. The reaction was initiated at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, and concluded with a standard melt-curve analysis which was used to confirm the specificity of subsequent qPCRs. Initially, qPCR products were visualized in a 1% agarose gel and sequenced as described above. In order to determine the efficiencies of each qPCR, a dilution series (1:5, 1:25, 1:125, 1:625, 1:3125) of a pooled cDNA sample was assayed and analysed as previously described (Pfaffl et al.,

Table 1
Reference sequence accession numbers, primer sequences, and annealing temperatures of PCRs used to amplify selected white rhinoceros mRNA targets.

Gene	NCBI accession No.		Primer sequence (5′-3′)		T[a] (°C)
	Horse	Rhinoceros	Forward	Reverse	
CCL4	XM_001503888	XM_004434846	GCACCAATGGGCTCAGAC	TCACAAAGTTGCGAGGAAGC	60
CXCL10	NM_001114940	XM_004430848	CACGTTTTCTGAGACACTGCTC	CAAGGGATCTTTCCACCTCT	60
IFNG	NM_001081949	XM_004429381	GTCCTTGGGACCTGATCAGC	TTGGGTGCAGTCACAGTTGT	65
YWHAZ	XM_001492988	XM_004431216	GAAAGGTCCCGGATGTTGCT	GGGGCTCAGCAGGCTCTG	65

[a] Annealing temperature.

Table 2
Reference sequence accession numbers, primer sequences, and selected parameters of RT-qPCRs for transcripts of the white rhinoceros.

Gene	Accession No.[a]	Primer sequence (5′-3′)		DMP[b] (◦C)	E[c] (%)	CV[d] (%)
		Forward	Reverse			
CCL4	MT465464; MT465465	CTCTCAGCACCAATGGGCTCAG	GCTTCCTCGCAACTTTGTGA	81	104	0.16
CXCL10	MT465462; MT465463	CCACGTGTTGAGATCATTGCC	AATTCTGGATGGTCTGGGACTC	76	112	0.22
IFNG	MT465460; MT465461	GAAGAACTGGAAAGAGGAGAGTG	TCCATGCTCTTTTGAATGACCTG	78.5	108	1.23
YWHAZ	MT465466	TGGTGACAAGAAAGGGATTG	ACAGAGAAGTTAAGGGCCAGAC	76.5	105	0.67

[a] NCBI Accession No.
[b] Derivative melt curve peak temperature.
[c] Amplification efficiency.
[d] intra-assay coefficient of variation.

2001). To validate the use of the relative quantification method, amplification efficiencies of *YWHAZ* and target genes were compared as previously described (Livak and Schmittgen, 2001). For each of the five *M. bovis*-infected and five uninfected rhinoceros, RT-qPCRs were performed in triplicate for the reference and each of the target genes using cDNA derived from blood incubated in QFT-Nil and QFT-TB antigen tubes.

2.6. Data analysis

To determine the intra-assay variability of replicate reactions of each qPCR, the coefficient of variation (CV) was calculated for each sample and reported as the mean of all samples (n = 20). For each sample, the relative expression of each target gene was normalised by subtracting the C_T value of *YWHAZ* from the C_T value of the target gene in order to calculate the relative abundance of the target gene mRNA for each sample (i.e. ΔC_T). Thereafter, the ΔC_T value derived from the QFT-Nil sample was subtracted from the ΔC_T value derived from the QFT-TB sample for all animals (i.e. $\Delta\Delta C_T$). The relative fold change ($2^{-\Delta\Delta CT}$) was used to derive a QFT GEA result as a measure of difference in expression of the target transcript in response to antigen stimulation (Livak and Schmittgen, 2001).

The GEA results for all target genes were then analysed in GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, California, USA). A Mann-Whitney *U*-test was used to determine if there was a difference in results for each gene between five *M. bovis*-infected and five uninfected rhinoceros. Results with a p-value < 0.05 were considered statistically significant.

3. Results

The RNA yields from blood incubated in the QFT-TB Antigen and QFT-Nil tubes ranged from 21 to 193 ng (mean of 52 ng) with a mean A260/A280 ratio of 1.84 (range: 1.53–2.09) and a mean A260/A230 ratio of 1.43 (range: 0.78–2.38).

For the equine RT^2 profiler assay, electrophoresis of RT-qPCR products revealed clear and reproducible bands of 80–200 base pairs for four of five reference genes, i.e. *ACTB*, *B2M*, *HPRT1* and *LOC100056766*. Duplicate assays of these genes showed a CV of $< 5\%$ and these four reference genes were therefore used to normalize relative expression calculations (Supplementary Table 1 and 2). In addition, C_T values of the RT^2 profiler controls were all within the manufacturer's recommended ranges (data not shown). The expression of 32 genes were validly measured for R1 (Supplementary Table 1) and 51 genes for R2 (Supplementary Table 2). Nine candidate target genes showed an upregulation greater than 2 fold in response to antigen stimulation (Table 3).

Two of these potential candidates were selected for further analysis using RT-qPCR assays, i.e. *CXCL10* as this has shown promise in numerous species and *CCL4* because it was identified in both R1 and R2. In addition, *IFNG* was included because there is a validated IGRA for TB diagnosis in white rhinoceros. Sequenced gene transcripts for *CCL4*, *CXCL10*, *IFNG* and *YWHAZ* showed high homology with published white rhinoceros sequences (Table 2).

All RT-qPCRs displayed low intra-assay variability with characteristic melt curves (data not shown) and melt peaks (Table 2). In addition, amplification efficiencies ranged from 104 to 112 % (Table 2) and the relative efficiencies of all target gene qPCRs were confirmed to be similar to the reference gene (data not shown). No products were obtained in the no-RT and no-template control reactions. Visualization following gel electrophoresis and sequencing of qPCR products confirmed the amplification of appropriate targets (data not shown).

In samples from *M. bovis*-infected rhinoceros, *CXCL10* showed a significant upregulation in response to antigen stimulation compared to samples from uninfected rhinoceros (Fig. 1). The median fold increases in antigen-specific *CXCL10* expression for the *M. bovis*-infected and uninfected animals were 20.5 and 1.6, respectively (Fig. 1). In contrast, no significant differences in *CCL4* and *IFNG* expression were seen between these groups (Fig. 1).

4. Discussion

This study describes the successful amplification of white rhinoceros cytokine gene transcripts from whole blood using the equine RT^2 profiler array. Using this assay, nine genes were identified as potential biomarkers of antigen recognition in blood incubated in the QFT stimulation system. In addition, novel RT-qPCR assays were developed to further evaluate these cytokine targets. Using this approach, measurement of *CXCL10* expression was confirmed to be able to distinguish between *M. bovis*-infected and uninfected white rhinoceros.

Seven of the genes identified by the equine RT^2 profiler array as displaying antigen-specific expression, i.e. *CCL4*, *CCL8*, *CSF3*, *CXCL10*,

Table 3
Selected equine RT^2 profiler assay results, for two white rhinoceros, using mRNA isolated from whole blood incubated in the TB and Nil tubes of the QuantiFERON® TB Gold (In-Tube) system.

Animal ID	Gene	QFT TB Tube (C_T)[a]			QFT Nil Tube (C_T)			Assay results	
		Target[b]	Reference[c]	ΔC_T	Target	reference	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
R1	*CCL4*	18.2	22.3	−4	19.1	21.4	−1.9	−2.1	4.3
	CCL8	30.7	22.3	8.5	32.2	21.4	11.2	−2.6	6.3
	IL23A	29.2	22.3	7	29.7	21.4	8.7	−1.7	3.2
	LTA	28.5	22.3	6.2	29.9	21.4	8.5	−2.2	4.7
	NODAL	29	22.3	6.7	30.2	21.4	8.7	−2.1	4.1
	TNF	25.7	22.3	3.4	26.2	21.4	5.2	−1.7	3.3
R2	*CCL4*	25.7	23.9	1.9	28.2	24.2	4.1	−2.2	4.7
	CSF3	34.3	23.9	10.5	37.6	24.2	13.5	−3	7.9
	CXCL10	33.8	23.9	10	36.3	24.2	12.2	−2.2	4.6
	GPI	28.1	23.9	4.2	30.1	24.2	5.9	−1.7	3.3

[a] Threshold Cycle.
[b] Mean C_T value of the target gene.
[c] mean C_T value of the reference genes.

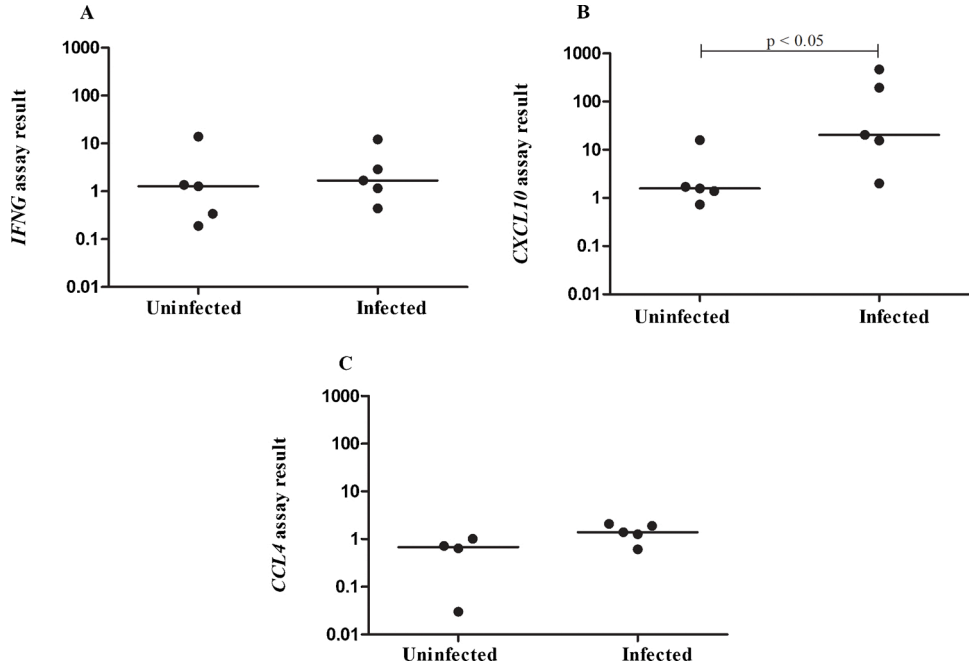

Fig. 1. Test results for the QuantiFERON® TB Gold (In-Tube) gene expression assays targeting *IFNG* (A), *CXCL10* (B) and *CCL4* (C) for *M. bovis*-infected and uninfected rhinoceros. The *CXCL10* assay results were significantly greater for *M. bovis*-infected buffaloes than for uninfected animals (Mann-Whitney *U*-test). Median assay results are shown by horizontal bars.

IL23A, *LTA* and *TNF* have been previously associated with both mRNA and protein biomarkers of mycobacterial infection and disease in humans and animals. In particular, *CXCL10* expression (encoding IP-10) is a highly sensitive diagnostic biomarker of TB in humans (Ruhwald et al., 2008) and other species (Roos et al., 2018; Bernitz et al., 2019) and is discussed, as is *CCL4*, in greater detail below. Similarly, antigen-induced *TNF* expression (detected as either mRNA or protein) is a TB biomarker common to humans and cattle but is diagnostic of disease severity rather than infection (Thacker et al., 2007; Sutherland et al., 2010; Wang et al., 2013). In contrast, while protein products of *CSF3* and *IL23A* expression have been identified as biomarkers of TB disease in humans (Sutherland et al., 2010; Wei et al., 2015), this is the first report of expression of these genes as candidate biomarkers in an animal species. However, the expression of *CSF3* and *IL23A* are both closely associated with that of *IL17* (Stark et al., 2005) which has been described as a diagnostic biomarker of *M. bovis* infection and disease in cattle (Blanco et al., 2011; Waters et al., 2016). The need for species-specific studies is highlighted by *CCL8* (which encodes the protein MCP-2), the expression of which has shown promise as a biomarker of *M. tuberculosis* infection in humans (Ruhwald et al., 2008) but not of *M. bovis* infection in African buffaloes (Goosen et al., 2014). Multiplex gene expression assays have the advantages of screening large numbers of potential target genes using limited samples and have been widely used for biomarker identification as well as diagnostic applications (Shukla et al., 2018; Palmer et al., 2020). Given the scarcity of samples from *M. bovis*-sensitized white rhinoceros and the lack of species-specific reagents, these findings support the use of the equine RT^2 profiler as a useful tool for screening analytes for the diagnosis of *M. bovis* infection in rhinoceros.

Of the candidate biomarkers selected for further evaluation by RT-qPCR, only antigen-specific *CXCL10* expression distinguished between *M. bovis*-infected and uninfected rhinoceros. This finding contrasts with reports that measurement of antigen-induced IFN-γ protein release is diagnostic of *M. bovis* infection in this species (Parsons et al., 2017; Chileshe et al., 2019). Similarly, measurement of *CXCL10* gene transcription, rather than that of *IFNG*, has shown promise as a diagnostic

biomarker in other animal species such as lions (Olivier et al., 2015) and spotted hyena (*Crocuta crouta*) (Higgitt et al., 2017). This may be related to differences in the kinetics of gene expression of these markers or the relative abundance of effector cells expressing these transcripts (Ruhwald et al., 2007). Importantly, the expression of *CXCL10* protein (IP-10) during antigen-induced immune activation is a sensitive and valuable biomarker of TB diagnosis in humans (Ruhwald et al., 2008), African buffaloes (Goosen et al., 2015), warthogs (Roos et al., 2018) and cattle (Parsons et al., 2016; Coad et al., 2019; Palmer et al., 2020). These studies strongly support the present findings that *CXCL10*/IP-10 should be further investigated as a diagnostic biomarker of *M. bovis* infection in rhinoceros.

Despite *CCL4* initially showing promise as a candidate biomarker of antigen recognition, no difference in *CCL4* responses was detected in QFT-processed samples from *M. bovis*-infected and uninfected rhinoceros. This gene encodes for MIP-1β, which, in combination with other cytokines, has been identified as a biomarker of TB disease in humans when measured in both unstimulated and antigen-stimulated blood (Chegou et al., 2009). However, MIP-1β levels are also elevated in unstimulated plasma from human patients with other respiratory diseases (Chegou et al., 2018) and antigen-stimulated samples of healthy controls (Wei et al., 2015). This might, in part, explain the discrepancy in the results obtained using the RT^2 profiler and RT-qPCR assays. While *CCL4* may display antigen-induced upregulation, the present findings do not support further investigation of this cytokine as a diagnostic marker of *M. bovis* infection in rhinoceros.

A major limitation for the optimal measurement of antigen-induced gene transcription of activated lymphocytes and other immune cells is the dynamic nature of this process. Gene expression may be induced directly by lymphocyte antigen recognition (e.g. *IFNG*), or indirectly in response to cytokine release (e.g. *CXCL10*), resulting in gene-specific expression kinetics (Abdalla et al., 2003). For this reason, sample incubation times must be optimized for assays designed to measure specific targets (Bibova et al., 2012). In cases where numerous gene transcripts are analysed, no single incubation time will be optimal, and multiple sampling time points post-infection can be of value (Palmer et al., 2020). This is supported by findings from the present study in which different targets were identified after 6 and 24 h of sample incubation.

In conclusion, despite limitations in this pilot study, the target gene *CXCL10,* identified by the RT^2 profiler, was confirmed as a promising biomarker by demonstrating significant upregulation in antigen-stimulated blood from *M. bovis*-infected rhinoceros in a novel cytokine GEA. This study demonstrates the potential that GEA may have for developing diagnostic tools for *M. bovis* infection in rhinoceros; future studies should investigate optimizing these assays in a larger cohort of rhinoceros.

Declaration of Competing Interest

On behalf of all the authors, I declare that none of us have known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2020.110168.

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