Veterinary Immunology and Immunopathology 162 (2014) 198–202

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Short communication

The evaluation of candidate biomarkers of cell-mediated immunity for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*)

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ARTICLE INFO

Article history:
Received 3 September 2014
Received in revised form 20 October 2014
Accepted 21 October 2014

Keywords:
African buffalo
Bovine tuberculosis
Enzyme linked immunosorbent assay
Interferon gamma
Interferon gamma-induced protein 10

ABSTRACT

We evaluated commercially available bovine enzyme linked immunosorbent assays (ELISA) and a human IP-10 ELISA to measure IP-10, MIG, MCP-1, MCP-2, MCP-3 and IL1-RA in buffalo plasma in order to identify sensitive markers of the immune response to *Mycobacterium bovis*-specific peptides. Additionally, we found that all coding mRNA sequences of these cytokines showed very high homology with their homologues in domestic cattle (97–99%) as did the derived amino acid sequences (97–99%). This high sequence homology between cattle and buffaloes supports the use of bovine ELISAs for the detection these cytokines in buffaloes. MCP-1 concentration showed a positive correlation with that of IFN-γ ($p = 0.0077$) and appears to occur in far greater abundance in buffaloes when compared to humans. Using a bovine IP-10 ELISA, levels of this cytokine were found to be significantly increased in antigen-stimulated blood samples from *M. bovis* test positive buffaloes ($p < 0.0001$) and IP-10 was detected in far greater abundance than IFN-γ. Measurement of IP-10 with this ELISA may prove to be a sensitive marker of *M. bovis* infection in African buffaloes.

1. Introduction

In Africa, buffaloes (*Syncerus caffer*) are one of the most important maintenance hosts of a variety of pathogens which cause disease in domestic cattle (Michel and Bengis, 2012). These include indigenous diseases such as foot-and-mouth disease, Corridor disease and alien diseases such as bovine brucellosis and bovine tuberculosis (BTB) (Michel and Bengis, 2012). In order to better control such diseases, the development of improved tests for their diagnosis and an understanding of their pathogenesis and epidemiology is essential.

In the case of BTB, the immunological diagnosis of infection relies primarily on the detection of a cell mediated immune response to *Mycobacterium bovis* antigens (Vordermeier et al., 2000). Examples of such assays include the single intradermal tuberculin skin test (SICTT) and the *in vitro* interferon gamma release assays (IGRA), the latter of which quantify antigen-stimulated release of this cytokine. Recently, the specificity of IGRAs have been improved with the availability of the Bovigam PC-EC assay (BEC) utilizing peptides simulating the *M. bovis* proteins 6 kDa early secretory antigenic target (ESAT-6) and the 10 kDa culture filtrate protein (CFP-10) and the Bovigam PC-HP assay (BHP) utilizing ESAT-6, CFP-10, peptides simulating Rv3615 and 3 additional mycobacterial antigens (Bass et al., 2013; Goosen et al., 2014). However, the sensitivity of BTB diagnosis in bovids remains suboptimal (Vordermeier

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http://dx.doi.org/10.1016/j.vetimm.2014.10.008
0165-2427/

Table 1
Enzyme linked immunosorbent assay kits and components, and buffalo plasma dilutions, used to measure selected cytokines in QFT-processed whole blood from African buffaloes.

Analyte	Plasma dilution[a]	Manufacturer	Product information
Bovine IFN-γ	1:2	Mabtech AB, Nacka Strand, Sweden	Kit no. 3115-1H-20
Bovine IP-10	1:10	Kingfisher Biotech Inc., St Paul, USA	Primary antibody: PB0385B-100 Secondary antibody: PBB0393B-050 Recombinant protein: RP0079B-005
Bovine MIG	1:2	Bethyl Laboratories Inc., Texas, USA	Kit no. E11-803
Bovine MCP-1	1:1000	Bethyl Laboratories Inc.	Kit no. E11-800
Bovine MCP-3	1:10	MyBioSource, California, USA	Kit no. MBS739771
Bovine MCP-2	1:10	MyBioSource	Kit no. MBS913274
Bovine IL1-RA	1:2	MyBioSource	Kit no. MBS740784
Human IP-10	1:2	PeproTech®, Rocky Hill, NJ	Kit no. 900-K39

[a] Final plasma assay dilution in each well.

et al., 2000) and it is possible that the quantification of biomarkers other than IFN-γ may improve the sensitivity of tests such as the BEC and BHP assays (Waters et al., 2003; Vordermeier et al., 2009; Jones et al., 2010; Blanco et al., 2011).

In humans, candidate biomarkers for the diagnosis of *Mycobacterium tuberculosis* infection include monocyte-derived chemokine IFN-γ-induced protein 10 (IP-10) (Ruhwald et al., 2009), monokine induced by interferon gamma (MIG) (Abramo et al., 2006; Chakera et al., 2011), monocyte chemoattractant protein (MCP)-1, MCP-2, MCP–3 and interleukin 1 receptor antagonist (IL1-RA) (Ruhwald et al., 2009; Chakera et al., 2011). Ruhwald et al. (2009) reported significantly higher concentrations of IP-10, IL1–RA, MCP-1, MCP-2 and MCP-3 compared to IFN-γ in antigen-stimulated blood samples from patients with active tuberculosis suggesting that all 5 biomarkers hold promise as immunological markers of tuberculosis in humans, with IP-10 and MCP-2 showing the greatest potential. Similarly, Abramo et al. (2006) showed a correlation between MIG concentration and IFN-γ production in 29 BCG-vaccinated controls and 24 TB patients, identifying MIG as a novel biomarker for *M. tuberculosis* infection. In cattle, Aranday-Cortes et al. (2012) reported that IP-10, MIG, Granzyme A and IL-22 mRNA expression show promise as biomarkers of antigen-induced immune responses which might be utilized for the diagnosis of *M. bovis* infection. However, using a human IP-10 ELISA, a study in cattle which were experimentally infected with *M. bovis* concluded that IP-10 is a poor diagnostic biomarker for the detection of this infection in this species (Waters et al., 2012).

The aim of this study was to evaluate the use of commercially available bovine ELISAs as well as a human IP-10 ELISA (Waters et al., 2012) for the measurement of selected candidate biomarkers of immune activation in response to *M. bovis* antigens in whole blood from African buffaloes.

2. Material and methods

2.1. Animals and sample preparation

In 2012, randomly selected buffaloes in the Hluhluwe–iMfolozi Park were captured and tested for *M. bovis* infection using the SICTT as previously described (Parsons et al., 2011). After SICTT assessment, whole blood (WB) was collected from all SICTT-positive buffaloes into sodium heparin tubes by jugular venipuncture. Immediately after collection, one ml of WB from each animal was transferred to a QFT TB Antigen tube (containing peptides simulating ESAT-6, CFP-10 and TB7.7) and a QFT Nil tube (containing saline), respectively. The QFT tubes were shaken vigorously, and within 4 h of WB transfer to the QFT tubes, these were incubated at 37 °C for 20 h, centrifuged at 3000 × *g* for 6 min and the plasma fraction stored at −80 °C. To identify positive QFT reactors, plasma samples harvested from the QFT tubes were used in a modified QFT (mQFT) assay using a commercially available bovine IFN-γ ELISA (Table 1) and a cut-off of 66 pg/ml, as previously described (Parsons et al., 2011). As previously reported, the mQFT assay is a highly specific test of *M. bovis* infection (Parsons et al., 2011, 2012; Goosen et al., 2014) and buffaloes that tested positive on both the SICTT and mQFT assays were considered to be infected for the purposes of this study.

2.2. Sequencing of selected buffalo cytokine mRNAs

Cytokine mRNA sequences of domestic cattle and sheep were obtained for IP-10, MIG, MCP-1, MCP-2, MCP-3 and IL1-RA from the Ensemble Genome Browser (http://www.ensembl.org/index.html). These mRNA sequences were aligned using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalo/) and highly homologous regions were used to generate primers with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Two forward and two reverse primers were designed, one of each within the untranslated regions flanking the translated region and one of each within the latter sequence (Table 2). Eight hundred ng of mRNA which had previously been extracted from *M. bovis* antigen-stimulated whole blood of two *M. bovis*-infected buffaloes (Parsons et al., 2012) was reverse transcribed using the QuantiTect reverse transcription (RT) Kit (Qiagen, Venlo, Netherlands) in a final volume of 80 μl. For each cytokine, each of the 4 primer combinations (0.4 μM of each primer) were used in a 25 μl PCR reaction containing 1 μl of cDNA, 2.5 μl of 10× buffer and 0.1 μl

Table 2
Sequencing data of selected cytokine mRNAs of the African buffalo: primers used for sequencing, NCBI Genbank accession numbers and homology with the equivalent sequences of domestic cattle.

Analyte	Primer sequence (5′–3′)				NCBI accession number	Homology with cattle (%)	
	External[a]		Internal[b]			mRNA (%)	Inferred amino-acid sequence (%)
	Forward	Reverse	Forward	Reverse			
IP-10	GCCTTTGCAAATATATACTGCATCT	ACTACGGTTTTCACCTACATTTCC	TCCTGCCCACGTGTCGAGAT	AGTCCACGGACAATTAGGGCTTGA	KM111562KM111563	98	97
MIG	GTGACTCAGTAGAACAAACACAGG	TAACACAAGATAGTGGTTGGTGA	AGGAATGGACGCTGTTCCTG	CTGGGTTTAGGCAGGCTTCA	KM111564KM111565	98	98
MCP-1	CCACCCTCTCGGTTTTCAAT	ATTCTTGCGAGGACACTTCC	AGGCCAAACCAGAGACCAAC	TATAGCAGCAGGCGACTTGG	KM111566KM111567	99	99
MCP-2	CCCATGCACTCTGCTCCCTATAA	TCCACTTACAGGAGCACTGA	CAGTTTCTACCCCAATCACCTG	AGACATCCCTGTCCGCTTTG	KM111558KM111559	99	98
MCP-3	AAGACTGCGAGCCCT GAGAA	ACTGGGACATCCCTTGCCAC	GCCCAGAT GACCCTCATGTC	GTCCACGTAGCCCAGCAC	KM111568KM111569	98	98
IL1-RA	CTCCAGGTCACAGGATGGAC	GCCACAGGGTGTGGACTTTA	GTTCAGAAACAGCCTGCCAC	TCTGGAGTCATGGACGTGCT	KM111560KM111561	98	99

[a] External primers annealing in the untranslated regions, flanking the translated region of interest.
[b] Internal primers annealing within the translated region of interest.

of HotStarTaq DNA polymerase (reagents from Qiagen) and 0.4 mM dNTPs (Promega Corporation, Fitchburg, WI, USA). The PCR reactions were initiated by incubation at 95 °C for 15 min and consisted of 45 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min after which PCR products were incubated for a further 10 min at 72 °C. These amplification products were sequenced with the 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's guidelines. Cytokine protein sequences were then inferred from cDNA sequences using the EMBOSS Transeq software (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). The degree of homology between all buffalo sequences and homologous sequences of cattle were calculated using ClustalW2 software.

2.3. Cytokine ELISAs

The concentration of IP-10, MIG, MCP-1, MCP-2, MCP-3 and IL1-RA in the QFT-processed plasma samples was measured using commercially available ELISAs (Table 1) according to the manufacturer's instructions. Furthermore, the absolute concentration of IP-10 in these samples was calculated with reference to a dilution series of recombinant human and bovine IP-10 for the human and bovine ELISAs, respectively (Table 1).

2.4. Statistical analysis

For each animal, the concentration of each analyte was characterized as the ELISA optical density (OD) and the antigen-dependent secretion of each analyte ($OD^{TB\text{-}Nil}$) was defined as the OD value of the QFT TB Antigen plasma sample (OD^{TB}) minus that of the QFT Nil plasma sample (OD^{Nil}). Using these OD values, a Wilcoxon signed-rank test was used to determine if the median concentration of each analyte was significantly different in plasma from the QFT Nil and TB Antigen tubes. Furthermore, absolute concentrations of antigen-specific IFN-γ and IP-10 were also compared using a Wilcoxon signed-rank test. The correlation between the OD^{TB} value for each analyte and that of IFN-γ was calculated as Spearman's correlation coefficient.

3. Results and discussion

The coding mRNA sequences of IP-10, MIG, MCP-1, MCP-2, MCP-3 and IL1-RA were obtained for two African buffaloes and submitted to the NCBI sequence database (http://www.ncbi.nlm.nih.gov/genbank/) (Table 2). The availability of these sequences should prove useful for the investigation of buffalo immunology by means of gene expression assays as previously described (Parsons et al., 2012). The sequences showed very high homology with those of cattle (97–99%) as did the inferred amino acid sequences (97–99%) (Table 2). This degree of homology between buffalo and cattle suggests that commercial bovine ELISA kits could be used to quantify these proteins in plasma samples from buffaloes.

These cytokines have been shown to be useful diagnostic biomarkers of cell activation following antigen recognition in humans (Abramo et al., 2006; Ruhwald et al.,

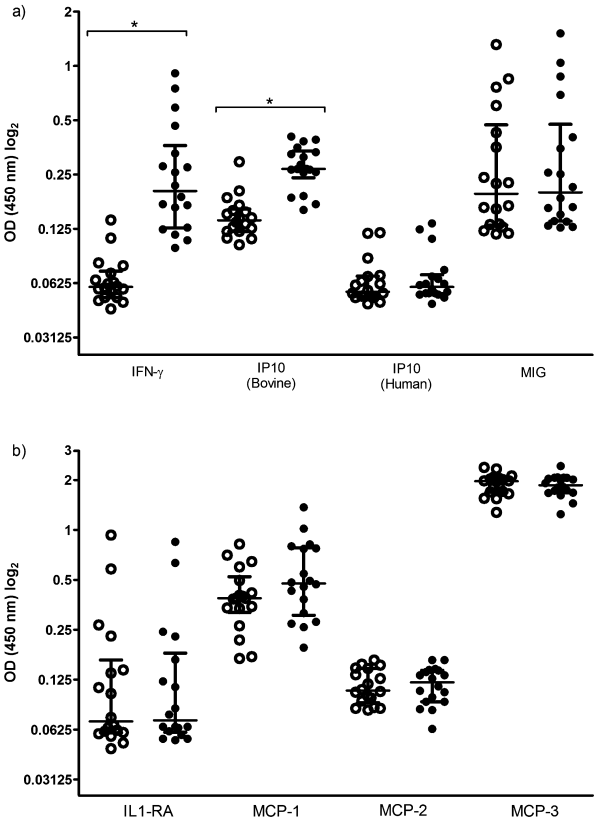

Fig. 1. Optical densities (including median and interquartile range) obtained in ELISAs of plasma samples harvested from QFT Nil tubes (○) and QFT TB Antigen tubes (•) following overnight whole blood incubation at 37 °C for (a) IFN-γ, IP-10, and MIG, and (b) IL1-RA, MCP-1, MCP-2 and MCP-3 (n = 18 SICTT-positive/mQFT-positive African buffaloes; * $p < 0.0001$).

2007, 2009). However, using commercial bovine ELISAs, only IFN-γ and IP-10 levels were found to be significantly increased in antigen-stimulated blood samples from SICTT-/mQFT-positive buffaloes (Figs. 1 and 2). Furthermore, MCP-1 was the only cytokine to show a significant

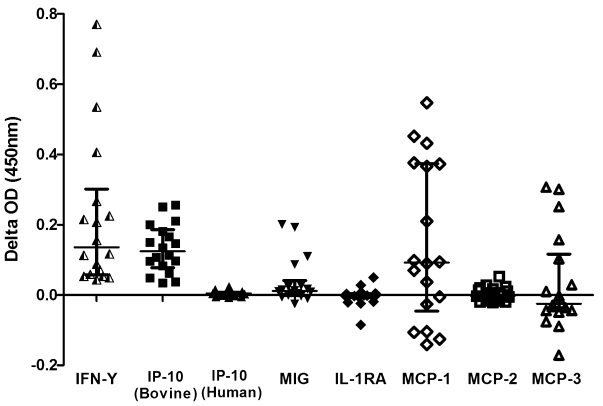

Fig. 2. The difference in plasma levels of IFN-γ, IP-10, MIG, IL1-RA, MCP-1, MCP-2 and MCP-3 harvested from QFT TB antigen tubes and QFT Nil tubes following overnight whole blood incubation at 37 °C (n = 18 SICTT-positive/mQFT-positive African buffaloes).

correlation with IFN-γ ($p = 0.0077$). The results for the remaining analytes were unexpected and we conclude that these are either poor indicators of antigen stimulation in buffaloes under the conditions described or that the bovine ELISAs did not efficiently detect these buffalo proteins. The latter is unexpected given the high degree of homology observed between cattle and buffalo transcripts of these analytes (Table 2). However, we cannot rule out that transcriptional, translational and post-translational differences between cattle and buffaloes may alter protein epitopes. Particularly surprising were the results for the human IP-10 ELISA which detected negligible levels of IP-10 in all buffalo plasma samples (0–137 pg/ml; Fig. 1). This is in contrast to findings from a study which used the same ELISA reagents to detect high levels of IP-10 in plasma from cattle following experimental infection with *M. bovis* (Waters et al., 2012). Our findings may indicate that the anti-human IP-10 antibodies used in this study have poor cross-reactivity to this protein of buffaloes despite their high predicted homology with cattle IP-10 (Table 2).

For the MCP-1 ELISA, plasma samples were diluted 1:1000. Nonetheless, MCP-1 ELISA signals were highly correlated with IFNg ($p = 0.0077$) suggesting that the results are a true reflection of MCP-1 abundance. Absolute concentrations of MCP-1 were not calculated; however, in humans, a typical plasma dilution factor for such an assay is 1:8 (Ruhwald et al., 2009) and our findings suggest that MCP-1 is particularly abundant in antigen-stimulated buffalo plasma. This cytokine is associated with a Th2 bias and enhanced production of IL-4 (Deshmane et al., 2009), both of which are associated with greater BTB pathology in cattle (Thacker et al., 2007). Moreover, an increase in MCP-1 plasma concentration due to a polymorphism in the MCP-1 gene promoter region has been shown to be associated with an increased susceptibility to pulmonary tuberculosis in humans (Flores-Villanueva et al., 2005). Our findings, therefore, warrant further investigation of the role of MCP-1 in buffalo BTB.

Using the bovine IP-10 ELISA, levels of this cytokine were shown to be elevated in antigen-stimulated samples in all SICTT-positive/mQFT-positive buffaloes (Figs. 1 and 2). In addition, antigen-specific IP-10 was released in significantly greater abundance than IFN-γ ($p < 0.0001$). This suggests that IP-10 may be at least as sensitive as IFN-γ as a diagnostic biomarker in buffaloes. Furthermore, because of its abundance, it may prove to be a more sensitive marker of *M. bovis* infection as has been shown for *M. tuberculosis* infection in humans (Ruhwald et al., 2007, 2009, 2011). The IP-10 molecule is also highly stable at room temperature (Aabye et al., 2011) and human plasma samples dried on filter membrane paper and transported via conventional postal service showed no loss of IP-10 signal (Aabye et al., 2013). This could prove particularly useful for the transport of buffalo samples from remote locations. Moreover, human studies have also shown *M. tuberculosis*-specific IP-10 responses to be less affected by co-infection with other bacteria and viruses, including HIV (Kabeer et al., 2010; Ruhwald et al., 2011). Again, this attribute might be particularly useful in buffaloes, a species which is known to be infected with a number of livestock pathogens (Michel and Bengis, 2012).

In conclusion, we have shown that IP-10 is a useful marker of immune activation by *M. bovis* antigens when using the bovine IP-10 ELISA. Our results highlight the limitations imposed on veterinary research by the lack of optimal immunological reagents and the need to interpret experimental results in this light. Our findings also suggest that the diagnostic potential of IP-10 for BTB in cattle be re-evaluated using species-specific reagents. Studies are currently under way to compare the sensitivity of IP-10 and IFN-γ as diagnostic biomarkers of *M. bovis* infection in African buffaloes.

Acknowledgments

We thank the National Research Foundation (NRF) and Medical Research Council (MRC) for financial support. Opinions expressed and conclusions arrived at are those of the authors and are not necessarily the views of the NRF or MRC. Furthermore, we acknowledge the Claude Leon Foundation and the NRF South African Research Chair Initiative in Animal Tuberculosis for personal funding and support. Lastly, we thank the Wildlife Capture Unit of Ezemvelo KZN-Wildlife for their assistance.

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