Veterinary Immunology and Immunopathology 173 (2016) 17–20

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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

Short communication

The stability of plasma IP-10 enhances its utility for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*)

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ARTICLE INFO

Article history:
Received 22 February 2016
Received in revised form 22 March 2016
Accepted 24 March 2016

Keywords:
African buffalo
Diagnosis
Interferon gamma-induced protein 10
Mycobacterium bovis
Protein saver cards

ABSTRACT

The measurement of interferon gamma-induced protein 10 (IP-10) in antigen-stimulated whole blood is a sensitive biomarker of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). However, this species often occurs in remote locations and diagnostic samples must be transported to centralised laboratories for processing. In humans, plasma IP-10 is highly stable and this feature contributes to its diagnostic utility; for this reason we aimed to characterize the stability of this molecule in buffaloes. Blood from *M. bovis*-infected and -uninfected animals was incubated with pathogen-specific peptides, saline and phytohaemagglutinin, respectively. Plasma fractions were harvested and aliquots of selected samples were: (i) stored at different temperatures for various times; (ii) heat treated before storage at RT, and (iii) stored on Protein Saver Cards (PSCs) at RT for either 2 or 8 weeks before measurement of IP-10. Incubation of plasma at 65 °C for 20 min caused no loss of IP-10 and this protein could be quantified in plasma stored on PSCs for 2 and 8 weeks. Moreover, for all storage conditions, IP-10 retained its excellent diagnostic characteristics. These features of IP-10 might allow for the heat inactivation of potentially infectious plasma which would facilitate the safe and simple transport of samples.

1. Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis (bTB), a zoonosis which can cause reduced productivity and death in a wide range of animals (Michel et al., 2006). In South Africa, African buffaloes (*Syncerus caffer*) are a major reservoir host of this pathogen and can act as a source of infection for cattle (Musoke et al., 2015) and other wildlife species (Olivier et al., 2015). The disease is therefore intensively controlled in buffaloes and this requires the immunodiagnosis of infected animals, primarily by the detection of cell-mediated immunity to *M. bovis* antigens (Vordermeier et al., 2000).

One such test is the interferon gamma (IFN-γ) release assay (IGRA) which detects the secretion of this cytokine by memory lymphocytes in response to *M. bovis* purified protein derivative (PPD) or antigens such as the 6-kDa early secreted antigenic target (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10) (Vordermeier et al., 2001; Waters et al., 2012). However, the applicability of this assay within the context of wildlife is confounded by the need for plasma cytokine concentrations to be measured with specialised equipment at centralised laboratories, while animals are often located in remote environments. This requires the transport of plasma under appropriately cooled or frozen conditions (Duncombe et al., 2013). Moreover, the movement of wildlife specimens risks the spread of highly infectious pathogens such as Foot and Mouth and African swine fever viruses (Duncombe et al., 2013).

As an alternative to IFN-γ, measurement of the antigen-specific release of IFN-γ-induced protein 10 (IP-10) has proven to be a strong diagnostic candidate in humans (Chakera et al., 2011; Ruhwald et al., 2007). Similarly, in buffaloes, the release of IP-10 in antigen-stimulated whole blood has been shown to be a useful marker of *M. bovis* infection (Goosen et al., 2014a) and to have a diagnostic sensitivity which is greater than conventional IGRAs (Goosen et al., 2015). In humans, plasma IP-10 is highly stable at 23 °C for up to 2 weeks (Aabye et al., 2012) and dried plasma, stored at 37 °C for 4 weeks on filter membrane Protein Saver Cards (PSCs) showed no decrease in IP-10 concentration (Aabye et al., 2012). This characteristic of IP-10 could have particular utility for veteri-

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http://dx.doi.org/10.1016/j.vetimm.2016.03.013
0165-2427/

nary samples as plasma stored on PSCs might easily be transported without any loss of diagnostic accuracy.

The aim of this study was, therefore, to investigate the diagnostic performance of assays measuring antigen-specific IP-10 for the diagnosis of *M. bovis* in African buffaloes following storage of plasma under various conditions.

2. Materials and methods

2.1. Animals

African buffaloes with known *M. bovis* exposure were captured, as previously described (Goosen et al., 2014b), in 2013 (Herds A13 and B13) and 2015 during a BTB test-and-slaughter program in the Hluhluwe-iMfolozi Game Reserve, South Africa. Buffaloes were tested using the tuberculin skin test (TST), as previously described (Goosen et al., 2014b; Parsons et al., 2011), as well as the mQFT assay, as described below. Animals were classified as either BTB-positive (TST and mQFT-positive) or BTB-negative (TST and mQFT-negative) and assigned to two groups. Group 1 consisted of all BTB-positive buffaloes (n = 15) and 19 randomly selected BTB-negative animals tested in 2015. Group 2 consisted of all BTB-positive buffaloes (n = 17) and 20 randomly selected BTB-negative animals from Herd B13. Ethical approval for the capture and testing of these buffaloes was granted by the Stellenbosch University Animal Care and Use Committee.

2.2. Whole blood stimulation

Ten ml of heparinized whole blood was collected, by venepuncture of the jugular vein, from each animal. 1 ml of blood was incubated for 20 h at 37 °C in each of the following QuantiFERON®-TB Gold In-Tube assay (QFT) blood collection tubes (Qiagen, Venlo, Limburg, Netherlands): a Nil tube containing saline and a TB Antigen tube containing ESAT-6, CFP-10 and TB7.7 peptides. Additionally, 1 ml of blood was incubated with phytohaemagglutinin (PHA) (Sigma-Aldrich, St. Louis, Missouri, USA) at a final concentration of 10 μg/ml. Thereafter, tubes were centrifuged at 1500 *g* for 6 min and plasma was harvested from each tube and stored at −80 °C. Plasma IFN-γ concentrations were determined using a bovine IFN-γ enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (kit 3115-1H-20; Mabtech, AB, Nacka Strand, Sweden). The mQFT assay result was defined as the IFN-γ concentration in plasma from the Nil tube subtracted from that from the TB Antigen tube. Animals with an assay result greater than 66 pg/ml were defined as mQFT-positive, as previously described (Parsons et al., 2011).

2.3. Heat treatment of plasma

Plasma derived from PHA-stimulated blood (activated plasma) and blood co-incubated with saline (non-activated plasma) was obtained from 8 randomly selected animals. Activated plasma was pooled, as was non-activated plasma, and aliquots of each pool were stored at −80 °C, 4 °C, room temperature (RT) and 37 °C for 0, 4, 8 and 12 days. Additionally, pooled activated plasma was incubated at 65 °C for 20 min and aliquots of heat-treated as well as untreated samples were stored at RT for 0, 1, 2, 3, 4, 7 and 14 days. For animals from Group 1, QFT-processed plasma was treated as follows: one aliquot of 30 μl was incubated at 65 °C for 20 min while a second aliquot was stored at −80 °C.

2.4. Storage of plasma on PSC

For animals from Group 2: 25 μl aliquots of QFT-processed plasma were spotted, in duplicate, onto 903 Protein Saver™ Cards (Whatmann plc, Maidstone, UK), dried at RT for 4 h, and stored at RT for 2 weeks (PSC-2w) and 8 weeks (PSC-8w), respectively. The remaining plasma was stored at −80 °C. Additionally, 5 aliquots of activated plasma (as described above) were spotted onto PSCs, as were 5 aliquots which had first been incubated at 65 °C for 20 min, and these were stored at RT for 7 days. All plasma samples spotted onto PSCs were stored in sealed plastic bags with a desiccant.

2.5. Measurement of IP-10 by ELISA

The concentration of IP-10 in plasma samples was measured using a commercial bovine IP-10 ELISA (Kingfisher Biotech Inc., St. Paul, MN, USA), as previously described in detail (Goosen et al., 2015), and recorded as either the relative concentration (ELISA optical density, OD) or as the absolute concentration of IP-10 (pg/ml). For plasma spotted onto PSCs, absolute amounts of IP-10 (pg) in samples was measured using this ELISA with the following modification: the sample comprised two circular discs punched out from the centre of the spot using a standard office paper punch (Carl Mfg. Illinois, USA, Inc.) and incubated in 100 μl of blocking buffer.

2.6. Statistical analysis

For plasma samples, diagnostic IP-10 test results were defined as the plasma concentration of IP-10 in blood incubated in the QFT TB Antigen tube minus that incubated in the Nil tube. Similarly, for the PSC-2w and PSC-8w samples, test results were defined as the difference in absolute amounts of IP-10 in the sample derived from the TB Antigen and Nil tubes. Using receiver operating characteristic (ROC) curve analysis, an optimal diagnostic cut off value for plasma samples stored at −80 °C was calculated by comparing test results for all BTB-positive (n = 32) and all BTB-negative buffaloes (n = 39). For BTB-positive and -negative animals from Group 2, cut off values were independently calculated for the PSC-2w and PSC-8w samples, respectively. In all cases, these values were calculated as the maximum value of Youden's index (Youden, 1950) and the diagnostic performance of each test was calculated as the area under the curve (AUC).

For BTB-positive animals from Group 1, the correlation between IP-10 test results derived from untreated and heat-treated plasma was described using Pearson's correlation coefficient and test results were compared using Student's *t*-test for paired samples. For BTB-positive buffaloes from Group 2, the correlations between the test results for the plasma IP-10, the PSC-2w and the PSC-8w assays were similarly calculated. Furthermore, PSC-2w and PSC-8w assay results for these animals were compared using student's *t*-test as was the mean concentration of IP-10 in activated plasma stored on PSCs, either with or without heat treatment.

The agreement between selected diagnostic results were calculated as Cohen's kappa coefficient (*k*) using freely available online software (http://graphpad.com/quickcalcs/kappa1/) and all other analysis was done using GraphPad Prism version 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results and discussion

3.1. Determination of a diagnostic cut off value for plasma IP-10

An optimal diagnostic cut off value of 1420 pg/ml (Fig. 1) for the plasma IP-10 assay resulted in a test sensitivity of 94% (95% CI: 79.2–99.2) and test specificity of 92% (95% CI: 79.1–98.4). This corresponds closely with an optimal IP-10 cut off value for QFT-processed samples from buffaloes which has previously been calculated as 1486 pg/ml (Goosen et al., 2015).