IP-10 is a sensitive biomarker of antigen recognition in whole blood stimulation assays used for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*).

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Running head: IP-10 for the diagnosis of *M. bovis* in buffaloes

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Abstract

African buffaloes (Syncerus caffer) are maintenance hosts of Mycobacterium bovis, the causative agent of bovine tuberculosis. They act as reservoirs of this infection for a wide range of wildlife and domestic species and the detection of infected animals is important to control the geographic spread and transmission of the disease. Interferon-gamma (IFN-γ) release assays utilizing pathogen-derived peptide antigens are highly specific tests of M. bovis infection; however, the diagnostic sensitivities of these assays are suboptimal. We evaluated the diagnostic utility of measuring antigen-dependent interferon gamma-induced protein 10 (IP-10) release as an alternative to measuring IFN-γ. M. bovis-exposed buffaloes were tested using the Bovigam PC-EC and Bovigam PC-HP assays and a modified QuantiFERON TB-Gold (mQFT) assay. IP-10 was measured in the harvested plasma and was produced in significantly greater abundance in response to M. bovis antigens in Bovigam-positive than in Bovigam-negative animals. For each assay, using the Bovigam results as a reference, receiver operating characteristic curve analysis was done to determine diagnostically relevant cut off values for IP-10. Hereafter, mQFT test results derived from measurement of IP-10 and IFN-γ were compared and a larger number of Bovigam-positive animals were detected using IP-10 as a diagnostic marker. Moreover, using IP-10, agreement between the mQFT assay and the Bovigam assays was increased while the excellent agreement between the Bovigam assays was retained. We conclude that IP-10 is a sensitive marker of antigen recognition and that measurement of this cytokine in antigen-stimulated whole blood might increase the sensitivity of conventional IGRAs in African buffaloes.

Keywords: African buffalo, bovine tuberculosis, enzyme linked immunosorbent assay, interferon gamma-induced protein 10, gamma interferon
Introduction

*Mycobacterium bovis* is the causative agent of bovine tuberculosis (BTB) in a wide range of domestic animals and wildlife (1). BTB in cattle populations is intensively controlled in many countries as it can result in reduced productivity or death of infected animals and poses a serious zoonotic risk. In South Africa, the African buffalo (*Syncerus caffer*) is a maintenance host of *M. bovis* and the early detection of infected animals is important to control the transmission of the pathogen to other wildlife and domestic species and to prevent the geographic spread of this disease by translocation (2, 3).

The most sensitive method for diagnosing *M. bovis* infection is by detection of the host’s cell mediated immune response to pathogen-specific antigens (4). Examples of such tests are the *in vivo* tuberculin skin test (TST) and *in vitro* interferon gamma release assays (IGRAs). The latter detect the release of interferon gamma (IFN-γ) in whole blood or from isolated peripheral blood mononuclear cells (PBMCs) in response to *M. bovis* purified protein derivative (PPD) (5) or more specific antigens such as the 6 kDa early secreted antigenic target (ESAT-6) and the 10 kDa culture filtrate protein (CFP-10) (6, 7). Recently, IGRAs utilizing the latter antigens have been described and evaluated for the diagnosis of BTB in buffaloes, i.e. the modified QuantiFERON TB-Gold (mQFT) assay and the commercially available Bovigam PC-EC assay (PC-EC) and Bovigam PC-HP assay (PC-HP) (3, 8). Notably, the mQFT assay, which is highly practical for use under field conditions, and the highly specific PC-EC assay are both less sensitive than the less specific PC-HP IGRA (8). As such, the utility of the mQFT and PC-EC assays would be increased by improving the detection of immune sensitization to ESAT-6/CFP-10.
One possibility for this might be the detection of additional or alternative biomarkers other than IFN-γ. In humans, a number of immunological proteins have been investigated as potential markers of immune activation in response to antigenic peptides and IFN-γ-induced protein 10 (IP-10) has proven to be a very strong candidate for the diagnosis of infection with M. tuberculosis (9–11). Similarly, and in contrast to reports from cattle studies (5), in M. bovis-infected buffaloes, IP-10 is significantly elevated in whole blood stimulated with ESAT-6/CFP-10 and is produced in much greater abundance than IFN-γ, suggesting its potential as an alternative or ancillary biomarker to IFN-γ in this species (10).

The aim of this pilot-study was, therefore, to determine if the measurement of IP-10 in antigen-stimulated whole blood is a useful diagnostic marker in buffaloes and whether it could be used to increase the sensitivity of established IGRA s. Because the PC-EC and PC-HP assays have been shown to have a greater sensitivity than the TST in this species (8) and because human studies have solely investigated IP-10 responses to peptide antigens (11), we used the PC-EC and PC-HP assays as reference tests. Moreover, since mycobacterial culture is considered to be an imperfect gold standard of M. bovis infection (12) and because this data was not available for Bovigam-negative animals in this study, an optimal diagnostic cut off value for IP-10 could not be calculated. We therefore evaluated the diagnostic utility of IP-10 by calculating a diagnostically relevant cut off value for this cytokine and thereafter, using both IFN-γ and IP-10 as diagnostic markers, the agreement between the mQFT assay and the highly sensitive and specific Bovigam peptide assays.

Materials and methods

Animals
In 2013, two hundred and thirty-one buffaloes were captured during an annual BTB test-and-slaughter program in the Hluhluwe-iMfolozi Game Reserve (South Africa) as previously described (3). Of these, eighty-four buffaloes were randomly selected and following chemical immobilization as previously described (8), ten ml of heparinized whole blood was collected from each animal by jugular venipuncture. Ethical approval for the capture and testing of these animals was granted by the Stellenbosch University Animal Care and Use Committee.

**mQFT, PC-EC and PC-HP assays**

One ml of blood was transferred to a QFT Nil tube, containing saline, and a QFT TB Antigen tube, containing peptides simulating ESAT-6, CFP-10, and TB 7.7 (Qiagen, Venlo, Limburg, Netherlands), respectively. Tubes were mixed thoroughly and incubated for 20h at 37°C. After incubation, 150 μl of plasma was collected without centrifugation from each QFT tube. The concentration of IFN-γ in all plasma samples was measured using a bovine enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s recommendations (kit 3115-1H-20; Mabtech, AB, Nacka Strand, Sweden). For each animal, the IFN-γ concentration in the Nil tube was subtracted from that in the TB Antigen tube and a differential value greater than 66 pg/ml was defined as a positive mQFT test result, as previously determined (3).

Performance of the PC-EC and PC-HP assays (Prionics, Schlieren-Zurich, Switzerland) included the incubation of 250 μl of whole blood with 25 μl saline, a 25 μl solution of peptides simulating ESAT-6 and CFP-10 (PC-EC assay) and a 25 μl solution of these peptides together with peptides simulating Rv3615 and 3 additional *M. bovis* antigens (PC-HP assay), respectively, for 20h at 37°C. All further analyses were done according to the manufacturer’s instructions.

**Bovine IP-10 ELISA and IP-10 assays**
The concentration of IP-10 in all plasma samples was measured as follows: 100 µl of a 5 µg/ml solution of anti-bovine IP-10 capture antibody (Kingfisher Biotech Inc., St Paul, USA, Catalog number PB0385B-100) in phosphate buffered saline (PBS) was added to each well of a 96-well flat bottom polystyrene plate (NUNC, Roskilde, Denmark) and incubated overnight at 4 °C. Thereafter, and after each subsequent step, plates were washed with a wash buffer consisting of 0.05% Tween-20 (Sigma-Aldrich, St. Louis, USA) in PBS. Blocking buffer (200 µl), consisting of 0.1% bovine serum albumin (BSA) (Roche, Basel, Switzerland) and 0.05% Tween-20 in PBS, was added to each well and the plates were incubated for 1h at room temperature (RT). Then, aliquots of 25 µl of plasma in 75 µl blocking buffer as well as a dilution series (0 – 5000 pg/ml) of recombinant bovine IP-10 protein (Kingfisher, Catalog Number RP0079B-005) were incubated in duplicate wells for 2h at RT. Hereafter, the plates were incubated at RT for 1h with 100 µl/well of 0.01 µg/ml biotinylated anti-bovine IP-10 antibody (Kingfisher, Catalog Number PBB0393B-050) diluted in blocking buffer and subsequently at RT for 1h with 100 µl/well of a streptavidin-horse radish peroxidase solution (R&D Systems, Minneapolis, USA) diluted in blocking buffer. After a final wash, plates were incubated for 30 min at RT with 100 µl of 0.4 mg/ml o-phenylenediamine dihydrochloride/well (Sigma-Aldrich, St. Louis, USA). Optical densities of each of the wells was measured at 450 nm with a LT-4000 Microplate Reader (Labtech, Vienna, Austria) and the concentration of IP-10 in each well was calculated using the standard curve generated from the dilution series of recombinant bovine IP-10 protein.

For each diagnostic assay, the IP-10 test result was calculated as the IP-10 concentration in the antigen-stimulated sample minus the IP-10 concentration in the sample incubated with sterile PBS. IP-10 tests using plasma obtained from the mQFT assay, PC-EC assay and PC-HP assay were defined as the IP-10(QFT), IP-10(EC) and IP-10(HP) tests, respectively.
Statistical analysis

Animals which tested positive by either the PC-EC or PC-HP assay were defined as Bovigam-positive while animals which tested negative for both assays were defined as Bovigam-negative. IP-10(QFT), IP-10(EC) and IP-10(HP) test results for animals which were Bovigam-positive and Bovigam-negative were compared using the Wilcoxon signed-rank test. For each IP-10 test, an optimal diagnostic cut off value for discrimination between Bovigam-positive and Bovigam-negative animals was calculated using receiver operating characteristic (ROC) curve analysis. The relevant cut off value for each assay was determined by selecting the maximum value of Youden’s index (YI), i.e. sensitivity + specificity − 1, which corresponds to the point nearest the upper left corner on the ROC curve (13). These cut off values were used to define animals as IP-10 test-positive or -negative. The diagnostic performance of each IP-10 assay was calculated as the area under the curve (AUC) of the respective ROC curve. All analyses were done using GraphPad Prism version 5 (GraphPad Software, March 2007). Lastly, using GraphPad software (http://graphpad.com/quickcalcs/kappa1/), agreement between selected assays was calculated as Cohen’s kappa coefficient (κ).

Results

IGRA test results

Of 84 buffaloes tested, the mQFT, PC-EC and PC-HP assays identified 31 (37%), 40 (48%) and 42 (50%) test-positive animals based on IFN-γ values, respectively. Forty-four animals were defined as Bovigam-positive, and of these, the mQFT, PC-EC and PC-HP assays detected 71%, 91% and 95%, respectively (Table 1).

Antigen-induced IP-10 release in whole blood
In order to confirm IP-10 as a useful biomarker of antigen-dependent immune activation, the IP-10 test results for Bovigam-positive and -negative buffaloes were compared using the Wilcoxon signed-rank test. For Bovigam-positive and -negative animals, the median IP-10(QFT), IP-10(EC) and IP-10(HP) test results were 5458 pg/ml and 5 pg/ml, 10269 pg/ml and 671 pg/ml, and 6773 pg/ml and 734 pg/ml, respectively (Fig. 1). For all assays, the IP-10 test results were significantly greater for Bovigam-positive than -negative animals (p < 0.001; Fig. 1).

**IP-10 as a diagnostic biomarker**

Diagnostic cut off values for the IP-10(QFT), IP-10(EC) and IP-10(HP) tests were calculated by comparing IP-10 test results for Bovigam-positive and -negative buffaloes using ROC curve analysis (Fig. 2, Table 2). A cut off value of 1486 pg/ml for the IP-10(QFT) assay detected all mQFT-positive animals and an additional 10 responders of which 7 were Bovigam-positive. A cut off value of 2155 pg/ml for the IP-10(HP) assay detected 40/42 (95%) PC-HP-positive animals and an additional 5 animals of which 2 were IP-10(EC) positive and 1 which was PC-EC-positive. The IP-10(QFT), IP-10(EC) and IP-10(HP) tests detected 86%, 82% and 93% of the 44 Bovigam-positive animals (Table 1). Of the Bovigam-negative buffaloes, 32/40 (80%) tested negative for all IP-10 assays (Table S1).

In order to further characterise the diagnostic utility of IP-10, agreements between the IGRAs and IP-10 tests were determined using Cohen’s kappa coefficient. The agreement between the mQFT assay and the combined Bovigam assays (k = 0.69) was substantially lower than the agreement between these assays and the IP-10(QFT) test (k = 0.79) (Table 3). Furthermore, the excellent agreement between the PC-EC and PC-HP assays was retained when IP-10 was used as a diagnostic marker (Table 3).
Discussion

The most sensitive immunological tests of *M. bovis* infection measure cell-mediated immune responses to pathogen-specific antigens, i.e. delayed type hypersensitivity in the case of the TST and IFN-γ release in the case of IGRAs. The latter tests depend on the presence of circulating antigen-specific memory T lymphocytes; however, if these cells are present in low numbers, IFN-γ production may be below the threshold of detection. Nonetheless, IFN-γ concentrations as low as 10 pg/ml as well as other cytokines such as TNF-α, Interleukin (IL)-12 and IL-4 can induce activation of monocytes and neutrophils resulting in secretion of IP-10 by these cells (14–16). IP-10 can therefore to be used as a proxy for lymphocyte activation (9). In humans, IP-10 has been shown to be a sensitive diagnostic biomarker of antigen recognition in a whole blood system (17) and the present study indicates its diagnostic utility in buffaloes.

Firstly, IP-10 is produced in far greater amounts in whole blood in response to *M. bovis* antigen stimulation in Bovigam-positive buffaloes than in Bovigam-negative animals (Fig 1 and 2). In addition to confirming this cytokine as a sensitive biomarker of antigen-induced immune activation as previously reported in buffaloes (10), these findings indicate the diagnostic potential of this protein to distinguish between these animal groups. Similarly, in humans, IP-10 is strongly induced in whole blood from tuberculosis patients stimulated with *M. tuberculosis*-specific antigens (11).

Secondly, the diagnostic performance of IP-10 was demonstrated by ROC analysis and the calculation of relevant diagnostic cut off values for the IP-10 tests. These analyses returned AUC values greater than 0.92 for all IP-10 tests indicating the discriminatory power of this biomarker (Table 1). This finding is particularly noteworthy given the limitations of these analyses which were performed with results from a restricted number of animals. A more accurate calculation of
diagnostic cut off values would require many hundreds of animals in both the test-positive and test-negative groups (18). Also, for the purposes of our analyses we elected to use the purely objective measure of a relevant cut off value, i.e. the YI index. This method, however, does not account for the fact that, when compared to the reference assays, the IP-10 tests might reasonably be expected to correctly identify a greater number of truly infected animals. It is probable, therefore, that this approach would overestimate the appropriate cut off values and underestimate the apparent sensitivities of the IP-10 tests. The limitation of this analysis is suggested by the relatively high cut off value of 4557 pg/ml calculated in this way for the IP-10(EC) test. This value is significantly higher than that calculated for the IP-10(QFT) assay and consequently, the IP-10(EC) assay detected only 82% of Bovigam-positive animals. Conversely, the IP-10(QFT) test cut off value of 1486 pg/ml increased the detection of Bovigam-positive animals from 71% for the mQFT assay to 86% indicating the diagnostic utility of this biomarker.

Thirdly, agreement between the IP-10(QFT) test and the combination of Bovigam assays ($k = 0.79$) was substantially greater than that between the latter result and the mQFT assay ($k = 0.69$). To a large degree, this increase in agreement is as a result of the increased sensitivity of the IP-10(QFT) test (Table 1). However, in addition to being a measure of agreement between positive test results, the kappa statistic measures agreement between negative test results and as such, the increased $k$-value in this case is also highly suggestive that an optimal IP-10 cut off value might not significantly compromise diagnostic specificity. Moreover, the diagnostic value of IP-10 is further indicated given that the excellent agreement between the PC-EC and PC-HP assays ($k = 0.86$) is retained when using IP-10 as a biomarker ($k = 0.81$).

A number of limitations of this pilot-study preclude definitive conclusions on the diagnostic utility of IP-10 in buffaloes. As mentioned, the limited sample size may have influenced the
calculation of appropriate IP-10 test cut off values. Moreover, the utility of IP-10 was evaluated using antigen-specific whole blood incubation assays which have been optimized for the measurement of IFN-γ and alternative incubation periods might be more appropriate for the measurement of IP-10. Also, Bovigam-negative animals which tested positive with the IP-10 tests were not slaughtered and no further immunological investigation was possible to confirm these results.

In conclusion, while recent advancements have been made in increasing the specificity of tests of CMI for the diagnosis of *M. bovis* infection in African buffaloes (3, 8) there remains a need for increased diagnostic sensitivity. This pilot-study indicates that IP-10 shows promise as a diagnostic biomarker in this species and that its measurement in peptide-stimulated whole blood may increase the sensitivity of conventional IGRAs. Future investigations of the diagnostic utility of this protein should include the validation of optimal diagnostic cut off values, the determination of its diagnostic sensitivity and specificity and an investigation of the diagnostic performance of IP-10 in combination with IFN-γ.

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**References**


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MCP-1, MCP-2, MCP-3, and IL-1RA hold promise as biomarkers for infection with M.

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Figure 1 IP-10 test results (M. bovis-specific IP-10 release) for Bovigam-positive and Bovigam-
negative buffaloes following processing of whole blood with the (a) mQFT, (b) PC-EC and (c)
PC-HP assays showing median and interquartile ranges. IP-10 release was significantly greater in
Bovigam-positive animals. * p < 0.001.

Figure 2 Receiver operating characteristic curves indicating the performance of selected cut off
values in order to differentiate between Bovigam-positive and Bovigam-negative buffaloes by
measurement of IP-10 in plasma obtained following processing of whole blood with (a) the
mQFT, (b) the PC-EC and (c) the PC-HP assays. The high agreement between the Bovigam and
IP-10 assays is indicated by the large area under the curve (AUC).

Table 1
The number and percentage of Bovigam-positive animals (n = 44) detected by measuring *M. bovis*-specific IFN-γ and IP-10 following the processing of whole blood with the mQFT, PC-EC and PC-HP assays.

**Table 2**

Receiver operating characteristic curve analysis of the IP-10(QFT), IP-10(EC) and IP-10(HP) tests for discrimination between Bovigam-positive (n = 44) and Bovigam-negative (n = 40) buffaloes.

**Table 3**

Test agreement (k and 95% confidence interval estimates) between the mQFT, PC-EC and PC-HP assays and the IP-10(QFT), IP-10(EC) and IP-10(HP) tests in a cohort of Bovigam-positive (n = 44) and Bovigam-negative (n = 40) buffaloes.
a) IP-10 (QFT) assay

AUC = 0.93

Sensitivity vs. 100% - Specificity%
c) IP-10(HP) assay

AUC = 0.93
Table 1

The number and percentage of Bovigam-positive animals (n = 44) detected by measuring *M. bovis*-specific IFN-γ and IP-10 following the processing of whole blood with the mQFT, PC-EC and PC-HP assays.

<table>
<thead>
<tr>
<th>Assay</th>
<th>No (%) of positive cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN-γ</td>
</tr>
<tr>
<td>mQFT</td>
<td>31 (71)</td>
</tr>
<tr>
<td>PC-EC</td>
<td>40 (91)</td>
</tr>
<tr>
<td>PC-HP</td>
<td>42 (95)</td>
</tr>
</tbody>
</table>

Table 2

Receiver operating characteristic curve analysis of the IP-10(QFT), IP-10(EC) and IP-10(HP) tests for discrimination between Bovigam-positive (n = 44) and Bovigam-negative (n = 40) buffaloes.

<table>
<thead>
<tr>
<th>IP-10 test</th>
<th>cut-off (pg/ml)</th>
<th>Sensitivity % (95% CI)</th>
<th>Specificity % (95% CI)</th>
<th>AUCa</th>
</tr>
</thead>
<tbody>
<tr>
<td>mQFT</td>
<td>1486</td>
<td>86.4 (72.7-94.8)</td>
<td>92.5 (79.6-98.4)</td>
<td>0.93</td>
</tr>
<tr>
<td>PC-EC</td>
<td>4557</td>
<td>81.8 (67.3-91.8)</td>
<td>92.5 (79.6-98.4)</td>
<td>0.93</td>
</tr>
<tr>
<td>PC-HP</td>
<td>2155</td>
<td>93.2 (81.4-98.6)</td>
<td>90 (76.4-97.2)</td>
<td>0.93</td>
</tr>
</tbody>
</table>

a Area under the curve.
Table 3

Test agreement (k and 95% confidence interval estimates) between the mQFT, PC-EC and PC-HP assays and the IP-10(QFT), IP-10(EC) and IP-10(HP) tests in a cohort of Bovigam-positive (n = 44) and Bovigam-negative (n = 40) buffaloes.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Bovigam</th>
<th>PC-HP</th>
<th>IP-10(HP)</th>
<th>PC-EC</th>
<th>IP-10(EC)</th>
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</thead>
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<tr>
<td>mQFT</td>
<td>0.69 (0.55 to 0.84)</td>
<td>0.74 (0.60 to 0.88)</td>
<td>n.d.</td>
<td>0.69 (0.53 to 0.84)</td>
<td>n.d.*</td>
</tr>
<tr>
<td>IP-10(QFT)</td>
<td>0.79 (0.65 to 0.92)</td>
<td>0.79 (0.65 to 0.92)</td>
<td>0.76 (0.63 to 0.90)</td>
<td>0.74 (0.59 to 0.88)</td>
<td>0.71 (0.56 to 0.86)</td>
</tr>
<tr>
<td>PC-HP</td>
<td>n.d.</td>
<td>1</td>
<td>0.83 (0.72 to 0.95)</td>
<td>0.86 (0.75 to 0.97)</td>
<td>0.79 (0.65 to 0.92)</td>
</tr>
<tr>
<td>IP-10(HP)</td>
<td>n.d.</td>
<td>0.83 (0.72 to 0.95)</td>
<td>1</td>
<td>0.79 (0.66 to 0.92)</td>
<td>0.81 (0.69 to 0.93)</td>
</tr>
<tr>
<td>PC-EC</td>
<td>n.d.</td>
<td>0.86 (0.75 to 0.97)</td>
<td>0.79 (0.66 to 0.92)</td>
<td>1</td>
<td>0.83 (0.71 to 0.95)</td>
</tr>
<tr>
<td>IP-10(EC)</td>
<td>n.d.</td>
<td>0.79 (0.65 to 0.92)</td>
<td>0.81 (0.69 to 0.93)</td>
<td>0.83 (0.71 to 0.95)</td>
<td>1</td>
</tr>
</tbody>
</table>

* not done