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)

Wynand J. Goosen, David Cooper, Michele A. Miller, Paul D. van Helden, Sven D. C. Parsons

DST/NRF Centre of Excellence for Biomedical Tuberculosis Research/SAMRC Centre for Tuberculosis Research/Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Stellenbosch, South Africa; Ezemvelo KZN Wildlife, St. Lucia, South Africa

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 (IGRAs) 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 levels. 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 cutoff values for IP-10. Thereafter, 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.

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 (IFN-γ) release assays (IGRAs). The latter detect the release of interferon gamma in whole blood or from isolated peripheral blood mononuclear cells (PBMCs) in response to M. bovis purified protein derivative (PPD) (5) or to 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, 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), have been described and evaluated for the diagnosis of BTB in buffaloes (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.
increase the sensitivity of established IGRA s. Because the PC-EC and PC-HP assays have been shown to have greater sensitivity than the TST in this species (8) and because human studies have solely investigated IP-10 responses to peptide antigens (10), 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 these data were not available for Bovigam-negative animals in this study, an optimal diagnostic cutoff value for IP-10 could not be calculated. We therefore evaluated the diagnostic utility of IP-10 by calculating a diagnostically relevant cutoff 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, 231 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, 84 buffaloes were randomly selected and, following chemical immobilization as previously described (8), 10 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. Blood (1 ml) was transferred to a QFT Nil tube containing saline solution 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 20 h 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) (kit 3115-1H-20; Mabtech, AB, Nacka Strand, Sweden) according to the manufacturer’s recommendations. 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 of saline solution, 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 20 h 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. A 100-μl volume of a 5 μg/ml solution of anti-bovine IP-10 capture antibody (catalog no. PB0385B-100; Kingfisher Biotech Inc., St. Paul, MN, USA) and 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, MO, USA) and PBS. Blocking buffer (200 μl), consisting of 0.1% bovine serum albumin (BSA) (Roche, Basel, Switzerland) and 0.05% Tween 20–PBS, was added to each well, and the plates were incubated for 1 h at room temperature (RT). Then, aliquots containing 25 μl of plasma and 25 μl blocking buffer as well as a dilution series (0 to 5,000 pg/ml) of recombinant bovine IP-10 protein (catalog no. RP0079B-005; Kingfisher) were incubated in duplicate wells for 2 h at RT. Thereafter, the plates were incubated at RT for 1 h with 100 μl/well of 0.01 pg/ml biotinylated anti-bovine IP-10 antibody (catalog no. PB0393B-050; Kingfisher) diluted in blocking buffer and subsequently at RT for 1 h with 100 μl/well of a streptavidin-horseradish peroxidase solution (R&D Systems, Minneapolis, MN, 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, MO, USA). Optical densities of each of the wells were measured at 450 nm with a LT-4000 Microplate Reader (Labsystem, Bottrop, Switzerland), 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 cutoff value for discrimination between Bovigam-positive and Bovigam-negative animals was calculated using receiver operating characteristic (ROC) curve analysis. The relevant cutoff 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 cutoff 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, Inc., La Jolla, CA, USA). Lastly, using online GraphPad software, agreement between selected tests was calculated as Cohen’s kappa coefficient (k) (GraphPad Software, Inc.).

RESULTS

IGRA 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 result values were 5,458 pg/ml and 5 pg/ml, 10,269 pg/ml and 671 pg/ml, and 6,773 pg/ml and 734 pg/ml, respectively (Fig. 1). For all assays, the IP-10 test result values were significantly higher for Bovigam-positive than -negative animals (P < 0.001; Fig. 1).

IP-10 as a diagnostic biomarker. Diagnostic cutoff 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 and Table 2). A

---

**TABLE 1** The number and percentage of Bovigam-positive animals 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.

| Assay   | No. (%) of positive cases (n = 44) |
|---------|-----------------------------------|
| IFN-γ   | 31 (71)                           |
| PC-EC   | 40 (91)                           |
| PC-HP   | 42 (95)                           |
| IP-10   | 38 (86)                           |
|         | 36 (82)                           |
|         | 41 (93)                           |
cutoff value of 1,486 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 cutoff value of 2,155 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 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 (see Table S1 in the supplemental material).

In order to further characterize 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 those 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, when these cells are present in low numbers, IFN-γ pro-
TABLE 2 Receiver operating characteristic curve analysis of the results 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

| IP-10 test | Cutoff value (pg/ml) | % sensitivity (95% CI) | % specificity (95% CI) | AUC |
|------------|----------------------|------------------------|------------------------|-----|
| mQFT       | 1,486                | 86.4 (72.7–94.8)       | 92.5 (79.6–98.4)       | 0.93|
| PC-EC      | 4,557                | 81.8 (67.3–91.8)       | 92.5 (79.6–98.4)       | 0.93|
| PC-HP      | 2,155                | 93.2 (81.4–98.6)       | 90 (76.4–97.2)         | 0.93|

* CI, confidence interval; AUC, area under the curve.

.. would overestimate the appropriate cutoff values and underestimate the apparent sensitivities of the IP-10 tests. The limitation of this analysis is suggested by the relatively high cutoff value of 4,557 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; consequently, the IP-10(EC) assay detected only 82% of Bovigam-positive animals. In contrast, the IP-10(QFT) test cutoff value of 1,486 pg/ml increased the detection of Bovigam-positive animals from 71% for the mQFT assay to 86%, indicating the diagnostic utility of this biomarker.

TABLE 3 Agreement 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

| Assay     | Bovigam | PC-HP | IP-10(HP) | PC-EC | IP-10(EC) |
|-----------|---------|-------|-----------|-------|-----------|
| mQFT      | 0.69 (0.53 to 0.84) | 0.74 (0.60 to 0.88) | ND*       | 0.69 (0.53 to 0.84) | ND       |
| IP-10(QFT) | 0.79 (0.65 to 0.92) | 0.79 (0.65 to 0.92) | 0.76 (0.63 to 0.90) | 0.74 (0.59 to 0.88) | 0.71 (0.56 to 0.86) |
| PC-HP     | ND      | 1     | 0.83 (0.72 to 0.95) | 0.86 (0.75 to 0.97) | 0.79 (0.65 to 0.92) |
| IP-10(HP) | ND      | 0.83 (0.72 to 0.95) | 1         | 0.79 (0.66 to 0.92) | 0.81 (0.69 to 0.93) |
| IP-10(EC) | ND      | 0.79 (0.65 to 0.92) | 0.81 (0.69 to 0.93) | 0.83 (0.71 to 0.95) | 1         |

* ND, not done.
ificity and of the diagnostic performance of IP-10 in combination with IFN-γ.

ACKNOWLEDGMENTS

We thank the National Research Foundation (NRF) and South African Medical Research Council (SAMRC) for financial support. Furthermore, we acknowledge the Claude Leon Foundation and the NRF South African Research Chair Initiative in Animal Tuberculosis for personal funding and support. Opinions expressed and conclusions arrived at are ours and are not necessarily the views of the NRF or MRC.

REFERENCES

1. Michel AL, Bengis RG, Keet DF, Hofmeyr M, de Klerk LM, Cross PC, Jolles AE, Cooper D, Whyte IJ, Buss P, Godfraud J. 2006. Wildlife tuberculosis in South African conservation areas: implications and challenges. Vet Microbiol 112:91–100. http://dx.doi.org/10.1016/j.vetmic.2005.11.035.

2. Grobler DG, Michel AL, De Klerk LM, Bengis RG. 2002. The gamma-interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (Syncerus caffer) in the Kruger National Park. Onderstepoort J Vet Res 69:221–227.

3. Parsons SDG, Cooper D, McCald A, McCall WA, Streicher EM, Le Maitre NC, Müller A, Gey van Pittius NC, Warren RM, van Helden PD. 2011. Modification of the QuantIFERON-TB Gold (In-Tube) assay for the diagnosis of Mycobacterium bovis infection in African buffaloes (Syncerus caffer). Vet Immunol Immunopathol 142:113–118. http://dx.doi.org/10.1016/j.vetimm.2011.04.006.

4. Vordermeier HM, Cockle PJ, Whelan AO, Rhodes S, Hewinson RG. 2000. Toward the development of diagnostic assays to discriminate between Mycobacterium bovis infection and bacille Calmette-Guérin vaccination. Clin Infect Dis 30(Suppl 3):S291–S298. http://dx.doi.org/10.1086/313877.

5. Waters WR, Thacker TC, Nonsense BJ, Palmer MV, Schiller I, Oesch B, Vordermeier HM, Silva E, Estes DM. 2012. Evaluation of gamma interferon (IFN-γ)-induced protein 10 responses for detection of cattle infected with Mycobacterium bovis: comparisons to IFN-γ responses. Clin Vaccine Immunol 19:346–351. http://dx.doi.org/10.1128/CVI.05657-11.

6. Vordermeier HM, Whelan A, Cockle PJ, Farrant L, Palmer N, Hewinson RG. 2001. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clin Diag Lab Immunol 8:571–578.

7. Bass KE, Nonsense BJ, Palmer MV, Thacker TC, Hardegger R, Schroeder B, Raebel AJ, Waters WR. 2013. Clinical and diagnostic developments of a gamma interferon release assay for use in bovine tuberculosis control programs. Clin Vaccine Immunol 20:1827–1835. http://dx.doi.org/10.1128/CVI.00519-13.

8. Goosen WJ, Miller MA, Chegou NN, Cooper D, Warren RM, van Helden PD, Parsons SDC. 2014. Agreement between assays of cell-mediated immunity utilizing Mycobacterium bovis-specific antigens for the diagnosis of tuberculosis in African buffaloes (Syncerus caffer). Vet Immunol Immunopathol 160:133–138. http://dx.doi.org/10.1016/j.vetimm.2014.03.015.

9. Chakera A, Bennett S, Cornwall R. 2011. A whole blood monokine-based reporter assay provides a sensitive and robust measurement of the antigen-specific T cell response. J Transl Med 9:143. http://dx.doi.org/10.1186/1479-5876-9-143.

10. Ruhwald M, Bjerregaard-Andersen M, Rabna P, Kofoid K, Eugen-Olsen J, Ravn P. 2007. CXCL10/IP-10 release is induced by incubation of whole blood from tuberculosis patients with ESAT-6, CFP10 and TB77. Microbes Infect 9:806–812. http://dx.doi.org/10.1016/j.micinf.2007.02.021.

11. Goosen WJ, Cooper D, Warren RM, Miller MA, van Helden PD, Parsons SDC. 2014. The evaluation of candidate biomarkers of cell-mediated immunity for the diagnosis of Mycobacterium bovis infection in African buffaloes (Syncerus caffer). Vet Immunol Immunopathol 162:198–202. http://dx.doi.org/10.1016/j.vetimm.2014.10.008.

12. de la Rua-Domenech R, Goodchild AT, Vordermeier HM, Hewinson RG, Christiansen KH, Clifton-Hadley RS. 2006. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculins tests, gamma-interferon assay and other ancillary diagnostic techniques. Res Vet Sci 81:190–210. http://dx.doi.org/10.1016/j.rvsc.2005.11.005.

13. Florkowski CM. 2008. Sensitivity, specificity, receiver–operating characteristic (ROC) curves and likelihood ratios: communicating the performance of diagnostic tests. Clin Biochem Rev 29:583–587.

14. Fang YS, Zhu LM, Sun ZG, Yu LZ, Xu H. 2012. Tumor necrosis factor-α pathway plays a critical role in regulating interferon-γ induced protein-10 production in initial allogeneic human monocyte-endothelial cell interactions. Transplant Proc 44:993–995. http://dx.doi.org/10.1016/j.transproceed.2012.03.051.

15. Bukowski RM, Rayman P, Molto L, Tannenbaum CS, Olenczi T, Peereboom D, Tubbs R, McLain D, Budd GT, Griffin T, Novick A, Hamilton TA, Finke J. 1999. Interferon-γ and CXC chemokine induction by interleukin 12 in renal cell carcinoma. Clin Cancer Res 5:2780–2789.

16. Méndez-Samperio P, Miranda E, Vázquez A. 2006. Expression and secretion of CXCL-8 and CXCL-10 from Mycobacterium bovis BCG-infected human epithelial cells: role of IL-4. Mediators Inflamm 2006:67451.

17. Ruhwald M, Bjerregaard-Andersen M, Rabna P, Eugen-Olsen J, Ravn P. 2009. IP-10, MCP-1, MCP-2, MCP-3, and IL-1RA hold promise as biomarkers for infection with M. tuberculosis in a whole blood based T-cell assay. BMC Res Notes 2:19. http://dx.doi.org/10.1186/1756-0500-2-19.

18. Flahault A, Cadilhac M, Thomas C. 2005. Sample size calculation should be performed for design accuracy in diagnostic test studies. J Clin Epidemiol 58:859–862. http://dx.doi.org/10.1016/j.jclinepi.2004.12.009.