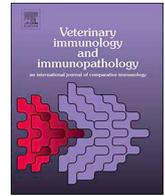




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Research paper

Impact of *Mycobacterium bovis*-induced pathology on interpretation of QuantiFERON®-TB Gold assay results in African buffaloes (*Syncerus caffer*)

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ABSTRACT

The cytokine interferon gamma-inducible protein 10 (IP-10) is a sensitive biomarker of *Mycobacterium bovis* (*M. bovis*) infection in African buffaloes (*Syncerus caffer*). However, elevated levels of IP-10 in QuantiFERON®-TB Gold (QFT) unstimulated whole blood compromises the utility of this biomarker. In this study, IP-10 and interferon gamma (IFN- γ) concentrations in whole blood samples from *M. bovis* culture-confirmed buffaloes with varying degrees of pathological changes ($n = 72$) and uninfected controls ($n = 70$) were measured in the IP-10 release assay (IPRA) and IFN- γ release assay (IGRA), respectively. Findings suggest that concentrations of both cytokines in QFT Nil tubes were higher in infected buffaloes with macroscopic pathological changes consistent with bovine tuberculosis compared to uninfected controls, and IGRA values increased with more severe pathological changes in infected buffaloes ($p < 0.05$). Finally, in culture-confirmed buffaloes with IPRA-negative and IGRA-positive test results, most animals were also those with the most advanced pathology. We conclude that IP-10 and IFN- γ concentrations measured in QFT Nil tubes may provide insight into the presence of *M. bovis* pathology in infected buffaloes. Furthermore, this study highlights the value in evaluating cytokine production in both antigen-stimulated and unstimulated samples when interpreting cytokine release assay results.

1. Introduction

Bovine tuberculosis (bTB) is a chronic inflammatory disease caused by the pathogen *Mycobacterium bovis* (*M. bovis*). *Mycobacterium bovis* has a wide mammalian host range including domestic animals, wildlife, livestock and humans (Dippenaar et al., 2017; Hlokwé et al., 2014). The disease is characterised by granulomas, formed when host immune cells infiltrate the tissue at the site of infection. Aggregates of infected macrophages fuse to form multinucleated giant cells followed by localized fibrosis and encapsulation (Palmer et al., 2007). These lesions are initially microscopic but with chronic inflammation, they increase

in size. Granulomas mature and multi-focal lesions may coalesce and become diffuse in the infected tissue, with caseous necrotic centres. Advanced bTB may result in morbidity and mortality (Domingo et al., 2014).

In South Africa, African buffaloes (*Syncerus caffer*) are wildlife reservoirs of *M. bovis* and thus efforts to control bTB disease in buffaloes are aimed at reducing infection in this species and spread to others. Since bTB is a chronic disease, buffaloes may present with clinical signs only after months or years of infection. Subsequently, strategies to identify *M. bovis*-infected buffaloes utilise measurement of early antigen-specific cell-mediated immune responses against *M. bovis*

Abbreviations: bTB, bovine tuberculosis; ELISA, enzyme-linked immunosorbent assay; IFN- γ , interferon gamma; [IFN- γ^{Nil}], concentration of interferon gamma in QFT Nil tube; IGRA, interferon gamma release assay; IP-10, interferon gamma-inducible protein 10; [IP-10^{Nil}], concentration of interferon gamma-inducible protein 10 in QuantiFERON®-TB Gold Nil tube; [IP-10^{TB}], concentration of interferon gamma-inducible protein 10 in QuantiFERON®-TB Gold TB antigen tube; IPRA, interferon gamma-inducible protein 10 release assay; L₁, lesion score 1; L₂, lesion score 2; L₃, lesion score 3; *M. bovis*, *Mycobacterium bovis*; *Mtb*, *Mycobacterium tuberculosis*; NVL, no visible lesions; QFT, QuantiFERON®-TB Gold

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antigens, rather than clinical disease. The early detection of infected buffaloes may enable management strategies to be implemented soon enough to limit the transmission of *M. bovis*.

In order to do this, *in vitro* cytokine release assays have been developed to quantify the production of cytokine biomarkers in plasma in response to antigen-specific whole blood stimulation. The QuantiFERON®-TB Gold (QFT) system is an easy-to-use stimulation platform comprised of a i) Nil tube containing saline (unstimulated control), ii) TB antigen tube containing early secretory antigen target 6 kD (ESAT-6) and culture filtrate protein 10 kD (CFP-10) (antigen stimulated) and iii) mitogen tube containing phytohemagglutinin (stimulated positive control). The interferon gamma-inducible protein 10 (IP-10) release assay (IPRA) and the interferon gamma (IFN- γ) release assay (IGRA) measure the cytokines IP-10 and IFN- γ in plasma, respectively, following QFT whole blood stimulation (Bernitz et al., 2019). The QFT IPRA and IGRA results are calculated as the plasma concentration of the cytokine in the QFT Nil tube subtracted from the plasma concentration of the cytokine in the QFT TB antigen tube. The QFT IPRA is highly sensitive and specific for detecting *M. bovis*-infection in buffaloes (Bernitz et al., 2019), however, the utility of the assay may be compromised by high concentrations of IP-10 in the QFT Nil tube ([IP-10^{Nil}]). This may cause false-negative test results in truly infected buffaloes and hinder the diagnostic performance of the QFT IPRA.

The phenomenon of elevated [IP-10^{Nil}] has been observed in humans and cattle and although not understood in the latter, has been associated with extent of disease in humans (Chen et al., 2011; Parsons et al., 2016). Therefore, the aim of this study was to improve interpretation of QFT IPRA and IGRA results in the context of the spectrum of bTB pathology. The [IP-10^{Nil}] and the concentration of IFN- γ in the QFT Nil tube ([IFN- γ ^{Nil}]) within groups of *M. bovis* culture-confirmed buffaloes with variable severity of lesions were compared to uninfected controls. Furthermore, the magnitude of QFT IPRA and IGRA values and discordant QFT assay results between groups of culture-confirmed buffaloes with different pathological features were compared.

2. Materials and methods

2.1. Animals

A total of 786 buffaloes were tested using the QFT IPRA and IGRA during bTB test-and-cull programmes in Hluhluwe iMfolozi Park, South Africa during 2016, 2017 and 2018. All test positive animals were culled and underwent post mortem examination as described below ($n = 145$), of which all animals confirmed positive on mycobacterial culture were used in this study ($n = 72$). During the same period, buffaloes from eight farms in South Africa with no known history of bTB were opportunistically tested with the QFT IPRA and IGRA when immobilised for reasons unrelated to this study. These buffaloes were used as uninfected controls ($n = 70$).

2.2. QFT IPRA and IGRA

Both cytokine release assays were performed on all buffaloes as previously described (Bernitz et al., 2019). Briefly, within eight hours of blood collection, 1 ml aliquots of heparinized whole blood were aseptically transferred to tubes of the QFT system (Qiagen, Venlo, Limburg, Netherlands); i) Nil; ii) antigen and iii) mitogen (respectively). Samples were inverted ten times and incubated for 20 h at 37 °C. Plasma was harvested following centrifugation. The cytokine IP-10 was measured using an in-house bovine-specific IP-10 enzyme-linked immunosorbent assay (ELISA; Kingfisher Biotech Inc., St Paul, MN, USA) and IFN- γ was measured using the commercially available cattletype® IFN- γ ELISA (Qiagen, Venlo, Limburg, Netherlands). The IPRA results were calculated as the [IP-10^{Nil}] subtracted from the concentration of IP-10 in the QFT TB antigen tube ([IP-10^{TB}]). An IPRA-positive result was defined as

a differential concentration of IP-10 ≥ 1486 pg/ml (Goosen et al., 2015). In contrast, the IGRA results were calculated as sample to positive control (S/P) ratios per the manufacturer's protocol; (OD of QFT antigen – OD of QFT Nil) / (OD cattletype® IFN- γ ELISA positive control – OD cattletype® IFN- γ ELISA negative control). A positive IGRA result was defined as S/P $\geq 35\%$. All *M. bovis*-uninfected buffaloes were test-negative on both the QFT IPRA and IGRA (data not shown).

2.3. Post mortem examinations and mycobacterial culture

Buffaloes that tested positive on one or both QFT assays were euthanised by gunshot and examined for gross bTB lesions during post mortem examination. Lymph nodes of the head and thoracic cavity and lungs were carefully dissected. Macroscopic pathological changes consistent with bTB were scored as follows: L₁, one small focal lesion (diameter < 10 mm); L₂, several small foci or a single lesion (diameter ≥ 10 mm and < 30 mm); and L₃, a single lesion (diameter ≥ 30 mm) or multifocal/confluent lesions (Palmer et al., 2007). In addition to the lesion score, a subjective description was recorded. To limit variation in lesion scoring, a single experienced veterinarian scored the lesions from all buffaloes. The lesion scores were used as a measure of pathological changes due to *M. bovis* infection. If an animal had no visible lesions (NVL), this was recorded, and pooled tissue samples of head (parotid, retropharyngeal, submandibular, and tonsils) and thoracic (mediastinal and tracheobronchial) lymph nodes were collected. Mycobacterial culture and genetic speciation were performed on all lesions and pooled tissue samples to confirm *M. bovis* infection as previously described (Goosen et al., 2014).

2.4. Statistical analyses

A Kruskal Wallance one-way ANOVA with a Dunn's post-test was used to compare i) [IP-10^{Nil}] and [IFN- γ ^{Nil}] in *M. bovis*-unexposed buffaloes and groups of culture-confirmed buffaloes with different lesion scores and ii) QFT IPRA and IGRA values in groups of culture-confirmed buffaloes with different lesion scores [GraphPad Prism version 5 software (GraphPad Software Inc., San Diego, CA, USA)]. Differences were considered statistically significant if $p < 0.05$. A Cochran's Q test with Bonferroni correction for multiple comparisons was used to compare the proportions of culture-confirmed buffaloes with concordant and discordant QFT assay results in each of the lesion score groups (MedCalc Free Trial Version 19.03 <https://www.medcalc.org/download.php>). Differences were considered statistically significant if $p < 0.0125$.

3. Results

Median [IP-10^{Nil}] were significantly higher in *M. bovis* culture-confirmed buffaloes with lesion scores of L₁, L₂ and L₃ compared to uninfected controls ($p < 0.001$; Fig. 1). Similarly, median [IFN- γ ^{Nil}] were significantly higher in animals with lesions (L₂, L₃) than uninfected controls ($p < 0.001$). Moreover, culture-confirmed buffaloes with the highest lesion score (L₃) had median [IFN- γ ^{Nil}] that were significantly higher than those with NVL ($p < 0.05$; Fig. 2).

There were no statistical differences in IPRA values between culture-confirmed buffaloes with different lesion scores (Fig. 3). However, median IGRA values for buffaloes with higher lesion scores (L₂ and L₃) were significantly higher than median values for buffaloes with NVL ($p < 0.001$) or a lesion score of L₁ ($p < 0.05$; Fig. 4).

The proportion of IPRA-positive IGRA-positive buffaloes in different lesion score groups were not significantly different. In contrast, the greatest proportion of IPRA-negative IGRA-positive buffaloes had the highest lesion score (L₃) and this was significantly greater than the proportion of animals with NVL or L₂ ($p = 0.002$). Furthermore, the greatest proportion of IPRA-positive IGRA-negative buffaloes had the lowest lesion score (L₁), although this was not significantly different

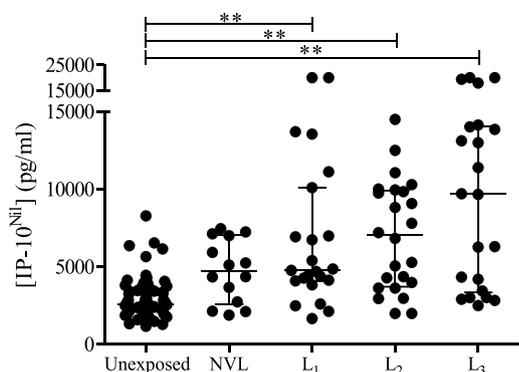


Fig. 1. The magnitude of IP-10 concentrations in unstimulated whole blood samples (QFT Nil tubes) from uninfected controls ($n = 70$) and *M. bovis* culture-confirmed buffaloes ($n = 72$) with no visible lesions (NVL) and lesion scores 1–3 (L_{1-3}). Horizontal bars represent median and interquartile ranges. P -values were calculated, and differences were considered statistically significant if $p < 0.05$ (** $p < 0.001$).

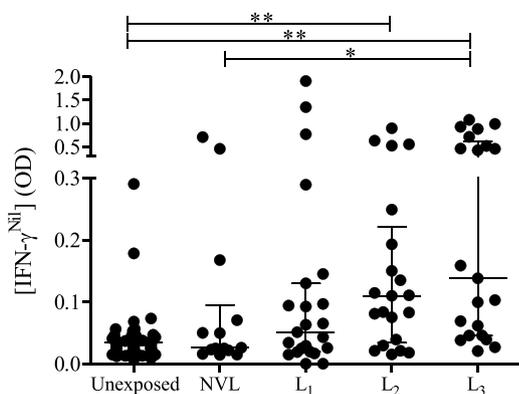


Fig. 2. The magnitude of IFN- γ concentrations in unstimulated whole blood samples (QFT Nil tubes) from uninfected controls ($n = 70$) and *M. bovis* culture-confirmed buffaloes ($n = 72$) with no visible lesions (NVL) and lesion scores 1–3 (L_{1-3}). Horizontal bars represent median and interquartile ranges. P -values were calculated, and differences were considered statistically significant if $p < 0.05$ (* $p < 0.05$ and ** $p < 0.001$).

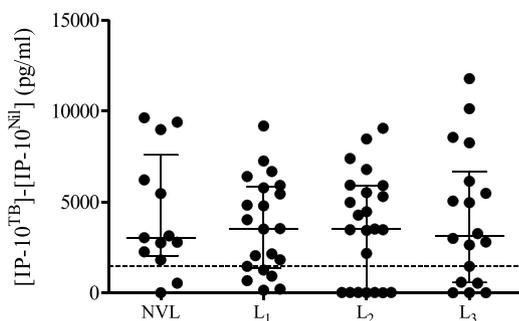


Fig. 3. The magnitude of QFT IPRA values in *M. bovis*-culture confirmed buffaloes with no visible lesions (NVL) and lesion scores 1–3 (L_{1-3}) ($n = 72$). The cutoff value for the assay is indicated by the dotted line (1486 pg/ml). Horizontal bars represent median and interquartile ranges. No statistical differences were observed between lesion score groups.

from the proportions of buffaloes with other lesion scores (Table 1).

4. Discussion

In this study, pathological changes were assessed by the presence and size of macroscopic bTB lesions in the lymph nodes and lungs of *M. bovis* culture-confirmed buffaloes. We evaluated antigen-stimulated and

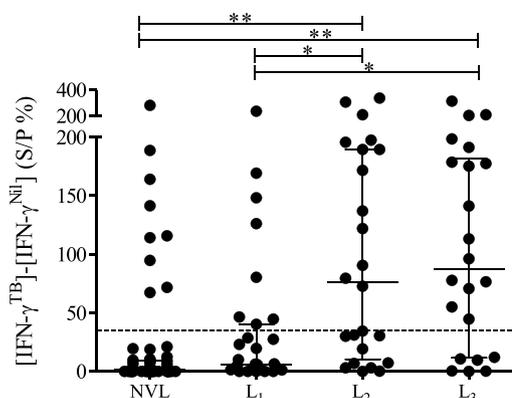


Fig. 4. The magnitude of QFT IGRA values in *M. bovis* culture-confirmed buffaloes with no visible lesions (NVL) and lesion scores 1–3 (L_{1-3}) ($n = 72$). The cutoff value for the assay is indicated by the dotted line (S/P = 35%). Horizontal bars represent median and interquartile ranges. P -values were calculated, and differences were considered statistically significant if $p < 0.05$ (* $p < 0.05$ and ** $p < 0.001$).

Table 1

Concordant and discordant QuantiFERON®-TB Gold assay results in *M. bovis* culture-confirmed ($n = 72$) buffaloes with no visible lesions (NVL) and lesion scores of 1–3 (L_{1-3}): L_1 , one small focal lesion (diameter < 10 mm); L_2 , several small foci or a single lesion (diameter ≥ 10 mm and < 30 mm); and L_3 , a single lesion (diameter ≥ 30 mm) or multifocal/confluent lesions. Superscript numbers denote lesion score groups that have statistically significant difference in results ($p = 0.002$).

Lesion score	IPRA ^a + IGRA ^c +	IPRA ^d IGRA +	IPRA + IGRA-
NVL	6	0 ¹	6
L_1	4	4	11
L_2	13	0 ²	8
L_3	8	8 ^{1,2}	4
Total	31	12	29

^a Interferon gamma-inducible protein 10 release assay.

^b Test positive.

^c Interferon gamma release assay.

^d Test negative.

unstimulated cytokine production to interpret the QFT IPRA and IGRA results in the context of bTB pathology in buffaloes.

Both the $[IP-10^{Nil}]$ and $[IFN-\gamma^{Nil}]$ were higher in buffaloes with culture-confirmed lesions than uninfected controls, and in culture-confirmed buffaloes, the $[IFN-\gamma^{Nil}]$ increased with lesion scores. This is consistent with studies showing the production of IP-10 and IFN- γ in unstimulated samples from human patients with active tuberculosis is higher than in uninfected controls, and unstimulated IFN- γ concentrations increase during the progression of active tuberculosis (Hasan et al., 2009). Additionally, both cytokines are included in an *ex-vivo* biomarker signature for the diagnosis of active tuberculosis in humans (Chegou et al., 2016; Chen et al., 2011; Hussain et al., 2010). Therefore, the $[IP-10^{Nil}]$ and $[IFN-\gamma^{Nil}]$ should be interpreted along with antigen-stimulated results, when analysing QFT cytokine release assay results to improve the detection of bTB in buffaloes.

In culture-confirmed buffaloes, QFT IGRA values increased as pathological changes increased. The cytokine IFN- γ is the archetypal measure of the Th1 immune pathway activated in response to *Mycobacterium tuberculosis* (*Mtb*) or *M. bovis* infection. These findings are consistent with studies in cattle, humans and European badgers (*Meles meles*) that have demonstrated a correlation between antigen-stimulated IFN- γ concentrations and disease severity (Sahiratmadja et al., 2007; Tomlinson et al., 2015; Vordermeier et al., 2002). Therefore, antigen-stimulated IFN- γ may not only be a biomarker of *M. bovis* infection but may provide information regarding the presence of bTB

Table 2

Interpretation of QuantiFERON®-TB Gold assay result permutations to detect infection and macroscopic pathology in buffaloes from a *Mycobacterium bovis*-endemic population.

IPRA ^a	IGRA ^b	Infection status	Further interpretation
Negative	Negative	Uninfected	No macroscopic pathology
Positive	Negative ^c	Early-infection	No or early macroscopic pathology
Positive	Positive	Infected	Early macroscopic pathology
Negative ^d	Positive	Chronically infected	Advanced macroscopic pathology

^a Interferon gamma-inducible protein 10 release assay.

^b Interferon gamma release assay.

^c IFN- γ may not yet be at detectable levels.

^d Due to elevated concentrations of IP-10 in QFT Nil tube.

pathology in buffaloes.

In contrast to the QFT IGRA, IPRA values did not differ among groups of culture-confirmed buffaloes with different lesion scores. We propose that high levels of antigen-stimulated IP-10 are produced in animals recently infected with *M. bovis* and so there is no marked difference between concentrations measured in culture-confirmed buffaloes with and without pathological changes. Supporting this, a study reported no significant differences between antigen-stimulated IP-10 in children with latent *Mtb*-infection and those with active disease (Whittaker et al., 2008).

In *M. bovis* culture-confirmed IPRA-positive IGRA-negative buffaloes, a greater proportion of animals presented with early signs of disease (L₁) compared to those with higher lesion scores. As discussed above, the cytokine IP-10 is a highly sensitive biomarker of infection and is suggested to detect buffaloes recently infected with *M. bovis*. The cytokine IP-10 is produced more than 2000-fold compared to IFN- γ (McInnis et al., 2005) and can therefore be measured at an earlier stage of infection when IFN- γ may not yet be detectable (Ruhwald et al., 2007). Therefore, we propose that IPRA-positive IGRA-negative buffaloes are animals that have recently been infected and this is supported by the findings of early lesions in these animals (Table 2). While IP-10 may not specifically be associated with development of pathological changes, this study confirms the use of IP-10 as a valuable biomarker of *M. bovis* infection in buffaloes.

In culture-confirmed IPRA-negative IGRA-positive buffaloes, a significantly greater proportion of animals had evidence of pathology compared to those with NVL. We propose that buffaloes with bTB lesions produce higher [IP-10^{Nil}] decreasing the IP-10 differential value between antigen-stimulated and unstimulated samples, resulting in an IPRA-negative result. This is supported by the observation that 5 of the 12 IPRA-negative IGRA-positive buffaloes in this study with bTB pathology, had higher [IP-10^{Nil}] compared to [IP-10^{TB}]. Thus, IPRA results with high [IP-10^{Nil}] should be regarded as inconclusive and not test-negative, whereas a buffalo that is IPRA-negative IGRA-positive should be suspected of having bTB pathology, if the [IP-10^{Nil}] is elevated (Table 2). An IPRA-negative (due to elevated [IP-10^{Nil}]) IGRA-positive result may have practical diagnostic relevance as a risk-based approach to culling the most infectious animals first, as bTB diseased animals are more likely to shed bacteria, thereby transmitting infection.

A limitation of this study includes the low sample numbers that may mask statistically significant differences. Moreover, in this study, lesion scores were used as a measure of pathological changes due to *M. bovis* infection. However, to investigate the extent of disease, additional criteria including body condition, dissemination of bTB to other organs and histopathology would need to be examined. Future studies should be performed in larger buffalo cohorts to determine cut-off values for the [IP-10^{Nil}] and [IFN- γ ^{Nil}] that may indicate *M. bovis* infection versus disease.

In conclusion, this study demonstrates that the magnitude of IP-10 and IFN- γ concentrations in QFT-processed whole blood can be used as an indicator of bTB pathology in buffaloes. Moreover, a negative QFT

IPRA result, resulting from high [IP-10^{Nil}], in combination with a positive QFT IGRA result, may be suggestive of the presence of disease due to *M. bovis* infection in buffaloes.

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest regarding the content of this article.

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