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Simultaneous measurement of antigen-induced CXCL10 and IFN-γ enhances test sensitivity for bovine TB detection in cattle☆

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ABSTRACT

Bovine tuberculosis (BTB) is a disease of economic and zoonotic importance caused mainly by *Mycobacterium bovis*. In addition to the tuberculin skin test, an interferon-gamma (IFN-γ) release assay (IGRA) blood test has been incorporated in the BTB control programs of numerous countries as an ancillary test to the skin test. A potential disadvantage of the IGRA assay is that it relies solely on the measurement of a single readout (i.e. IFN-γ) for the detection of BTB. In this study we have assessed the practical use of CXCL10 as an additional biomarker for the diagnosis of BTB in the setting of the current testing approach alongside IGRA. To do so, we have assessed both IFN-γ and CXCL10 readouts in blood cultures from a variety of different BTB cattle groups stimulated with standard tuberculin reagents and also with more specific defined antigens (ESAT-6, CFP-10 and Rv3615c). When using a tuberculin based whole blood assay, CXCL10 alone could not substitute for IFN-γ as the analyte measured in the test without reducing the sensitivity of detecting BTB animals. However, when used as an additional test readout, CXCL10 identified BTB animals that failed to induce IFN-γ responses. When tested in non-infected animals, the use of the dual biomarker system had the potential to lower overall test specificity, however this could be overcome by raising the cut-off values for CXCL10 test positivity. Taken together, the results demonstrate that in particular settings, measurement of CXCL10 has the potential to complement the current use of IFN-γ in blood assays to maximise the detection of BTB.

1. Introduction

Bovine tuberculosis (BTB) is a livestock disease of economic and zoonotic importance caused by pathogens of the *Mycobacterium tuberculosis* complex, mainly by *Mycobacterium bovis* (*M. bovis*). The global financial burden of BTB has been estimated at $3 billion US dollars annually (Waters et al., 2012a). In Great Britain (GB), the disease continues to be a challenge to the farming community and its control centres on the application of the single intradermal comparative cervical tuberculin (SICCT) skin test and the subsequent removal of animals found to be positive (reactors). In addition, an interferon-gamma (IFN-γ) release (IGRA) blood assay using avian and bovine tuberculin has been incorporated in the BTB control programs of numerous countries, including the UK, as an ancillary test to the standard skin test. Used in this way, IGRA is a useful tool in particular situations such as persistent TB herd breakdowns, severe TB herd breakdowns (as an alternative to whole herd slaughter), and confirmed new TB herd breakdowns outside of endemic BTB areas (de la Rua-Domenech et al., 2006).

Both the SICCT skin test and IGRA assays are conventionally based on the detection of a cell-mediated immune response to tuberculin test reagents, i.e. bovine purified protein derivative (PPD-B) and avian purified protein derivative (PPD-A). Given that *M. avium* subsp. *paratuberculosis* infection causing Johne's Disease (Alvarez et al., 2009; Aranaz et al., 2006) and immunisation with *M. avium* subsp. *paratuberculosis* vaccines (Kohler et al., 2001) can compromise the sensitivity and specificity of BTB detection when using tuberculin reagents, the use of *M. bovis*-specific antigens could mitigate the limitation of PPD in such settings. Indeed, from October 2017 the defined mycobacterial antigens ESAT-6 and CFP-10 have been used in GB to increase IGRA test sensitivity in situations such as herds co-infected with *M. avium* subsp. *paratuberculosis* (Shelley Rhodes, personal communication). Peptide cocktails based on these antigens, along with another cocktail covering the amino acid sequence of the Rv3615c antigen, also form the basis of a blood IFN-γ DIVA test (Differentiating Infected from Vaccinated Animals) capable of

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identifying *M. bovis* infected animals in the setting of BCG vaccination (Sidders et al., 2008; Vordermeier et al., 2001). These antigens can also be used as DIVA skin test antigens (Jones et al., 2012).

A potential disadvantage of the IGRA assay is that it relies on the measurement of a single readout (IFN-γ) for the detection of BTB. Therefore, identification of additional biomarkers for the diagnosis of BTB has been the focus of several studies. Analysis of mycobacterial antigen induced mRNA levels in cattle peripheral blood mononuclear cells (Aranday-Cortes et al., 2012; Blanco et al., 2011; Waters et al., 2012b) or whole blood (Xin et al., 2018) revealed greater expression of several cytokines/chemokines (including IL-6, IL-17 A, IL-22, CXCL9, CXCL10 and TNF-α) in samples from *M. bovis* infected animals compared to uninfected controls, suggesting that these may be useful diagnostic targets. Indeed, several human TB studies have demonstrated that CXCL10 (also known as interferon-gamma inducible protein-10; IP-10) can be used as a biomarker of TB, where significantly greater levels of mycobacterial antigen-induced CXCL10 protein were detected in whole blood of TB patients compared to healthy controls (Jenum et al., 2016; Wang et al., 2012, 2016; Whittaker et al., 2008). In contrast to measuring mRNA, initial studies quantitating CXCL10 protein levels in *M. bovis*-infected cattle were not promising (possibly due to the use of a human specific CXCL10 ELISA to quantitate protein levels), where responses to mycobacterial antigens did not generally exceed responses in non-stimulated cultures (Waters et al., 2012b). However, subsequent studies using a cattle specific CXCL10 ELISA have shown more promise. In BTB cattle, significantly greater CXCL10 responses were seen in whole blood cultures stimulated with mycobacterial antigens compared to non-stimulated cultures (Parsons et al., 2016). Furthermore, greater levels of mycobacterial antigen-induced CXCL10 protein were observed in whole blood samples from *M. bovis* infected cattle compared to uninfected controls (Parsons et al., 2016; Xin et al., 2018) and in IGRA positive African buffaloes compared to IGRA negative controls (Goosen et al., 2015). Although these studies have identified CXCL10 as a potential biomarker for BTB diagnosis, they have not addressed or demonstrated whether this provides any additional benefit over the current use of IGRA tests.

In this study we have assessed the practical use of CXCL10 as a biomarker for the diagnosis of BTB in the setting of the current testing approach using IGRA. To do so we have asked the following specific questions: (i) does CXCL10 provide a more sensitive readout for detecting BTB compared to IFN-γ; and (ii) can CXCL10 complement the IFN-γ assay to maximise the detection of BTB? We have assessed these readouts in blood cultures from a variety of different BTB cattle groups stimulated with standard tuberculin reagents and also the more specific defined antigens (e.g. ESAT-6, CFP-10 and Rv3615c).

2. Materials and methods

2.1. Animals

Blood and/or whole blood culture plasma supernatants were acquired from animals from several independent sources:

Group A: Blood from cattle naturally infected with BTB, identified as skin test reactors during routine UK BTB surveillance operations and recruited to APHA Weybridge (n = 20). All animals also tested positive in the tuberculin-based IGRA assay. Post-mortem examinations were performed on all these animals and *M. bovis* infection confirmed by pathology and/or culture.

Group B: Blood from TB negative cattle sourced from farms with no known history of BTB and in BTB-free areas, which were recruited to studies at APHA Weybridge (n = 55).

Group C: Blood from cattle naturally infected with BTB, identified as tuberculin IGRA positive cattle from a UK herd tested during routine UK BTB surveillance operations (n = 22).

Group D: Whole blood culture plasma supernatant from tuberculin IGRA positive cattle identified during routine UK BTB surveillance operations (supplied by APHA Scientific Laboratories Services routine diagnostic laboratories, n = 71).

Group E: Whole blood culture plasma supernatant from SICCT positive cattle in herds with current active outbreaks of BTB in the Republic of Ireland (n = 124).

Group F: Blood from cattle on BCG vaccination/*M. bovis* infection studies at APHA Weybridge (Dean et al., 2015; Jones et al., 2017) which were IFN-γ negative to the DIVA antigens ESAT-6/CFP10 and Rv3615c but confirmed as *M. bovis* infected by pathology and/or culture (n = 18).

2.2. Ethics statement

Cattle in groups A, B and F were experimental animals on studies conducted at APHA Weybridge. The animal procedures for these studies were approved by the Animal and Plant Health Agency (APHA) Animal Welfare and Ethical Review Body. For group E animals, the SICCT and collection of blood samples on which the blood IFN-γ tests were carried out was conducted as part of the Irish national BTB eradication programme, which is subject to the EU trade Directive 64/432/EEC, and which governs the nature and frequency of testing. Blood sample testing was approved by the UCD Animal Research Ethics Committee (AREC-E-16-34-Gormley).

2.3. Blood stimulation

With the exception of group E, heparinised blood samples from all other groups were stored overnight at room temperature before stimulation for 20 to 24 h at 37 °C in 5% CO_2 with the following: bovine and avian tuberculin (PPD-B and PPD-A; Prionics, Lelystad, Netherlands), PPD-B 300U/ml final concentration; PPD-A 250U/ml final concentration; positive mitogen control of pokeweed mitogen (Sigma, UK) at 10 μg/ml final concentration or a negative control of RPMI-1640 (Gibco, UK) alone. In some groups, blood samples were also stimulated with two specific-antigen peptide cocktails: ESAT-6/CFP-10 and Rv3615c, both at 5 μg/ml/peptide final concentration (synthetic peptides produced by Pepceuticals, Enderby, UK). After stimulation, blood was centrifuged at 300 g for 10 min and the plasma supernatant was harvested and stored at −80 °C until required.

For group E whole blood was collected into heparin tubes (BD Medica, Dublin, Ireland) and submitted to the laboratory within 24 h. Aliquots of the heparinised blood (1.5 ml) were dispensed into individual wells of 24-well tissue culture plates (Cruinn, Ireland) containing either PPD-B (20 μg/ml final concentration), PPD-A (10 μg/ml final concentration) for *in vitro* antigen stimulation or phosphate buffered saline (PBS) for a non-stimulated control. The tuberculin used for antigen stimulation was from the same production batch as for the SICCT (Thermo-Fisher Scientific, Lelystad, Netherlands). These cultures were incubated for 18 h at 37 °C in a humidified atmosphere with 5% CO_2. The stimulated blood was centrifuged at 1500 g for 15 min and the plasma supernatant was harvested and stored at −20 °C until required.

In all groups, the time delay between collecting and stimulating the blood samples was included so that the conditions in this study reflected as closely as possible the protocol for the current whole blood IGRA assay used in GB (i.e. next day testing). Blood processing and stimulation methods were consistent with the Bovigam® test kit instructions and the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2018.

2.4. Interferon-gamma release assay

The production of IFN-γ in each sample was measured using the Bovigam® test kit following manufacturer's instructions (Thermo Fisher Scientific). Samples were tested in duplicate and the IFN-γ concentrations were taken as the optical density (OD) reading at 450 nm as measured on a Multiskan Ascent (Thermo Labsystems) plate reader. Test results were taken as the PPD-B stimulated value minus the PPD-A

stimulated value (ΔPPD), or ESAT-6/CFP-10 and Rv3615c values minus the unstimulated control value. Results greater than or equal to 0.1 were taken as a positive response.

2.5. CXCL10 ELISA

CXCL10 in plasma samples was measured as follows. Nunc MaxiSorb flat-bottom 96 well plates were coated overnight at 4 °C with 100 µl/well anti-bovine CXCL10 polyclonal antibody (1 µg/ml, Kingfisher Biotech, St Paul, MN, USA) in carbonate-bicarbonate buffer pH9.6. Between subsequent steps, wells were washed with wash buffer (PBS with 0.05% Tween 20) and all further steps were undertaken at room temperature. Wells were blocked for 1 h with 200 µl/well PBS with 3% bovine serum albumin (BSA, Sigma). After blocking, plasma samples (diluted 1 in 2 in assay buffer, PBS with 1% BSA) were incubated in duplicate wells for 2 h (100 µl/well). After washing, plates were incubated for 1 h with 100 µl/well biotinylated anti-bovine CXCL10 polyclonal antibody (Kingfisher Biotech, 0.2 µg/ml in assay buffer). A further wash was followed by incubation for 30 min with 100 µl/well Streptavidin-horseradish peroxidase (GE Healthcare, UK) diluted 1:2000 in assay buffer. After a final wash step, 100 µl of Tetramethylbenzidine (Sigma) was added to each well for up to 15 min before colour reactions were stopped with 100 µl/well 0.5 M sulphuric acid. The OD of each reaction was measured at 450 nm and the concentration of CXCL10 in each sample was calculated by comparing the OD reading against a standard curve generated on each plate using a dilution series of recombinant CXCL10 (Kingfisher Biotech). Test results were taken as the PPD-B stimulated value minus the PPD-A stimulated value (ΔPPD), or ESAT-6/CFP-10 and Rv3615c values minus the unstimulated control value.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software Inc.). Cut-offs for differentiating between groups were determined using Receiver Operator Characteristic (ROC) curves. Comparison of test relative sensitivities was performed using the Fisher exact test using one-sided or two-sided analysis where appropriate.

3. Results

CXCL10 ELISA data from 20 field reactors with confirmed BTB infection (group A) and 55 non-infected cattle (group B) were subjected to ROC analysis to determine positive/negative cut-offs for stimulation with ΔPPD, ESAT-6/CFP-10 or Rv3615c peptide cocktails (Fig. 1). All three stimuli gave significant areas under the curve, suggesting that CXCL10 may show a potential as a readout cytokine capable of distinguishing between BTB infected and non-infected control animals when using these test reagents. As we were initially interested in seeing if CXCL10 is able to replace IFN-γ as a readout in blood tests for BTB, cut-offs for CXCL10 positivity at a predetermined specificity of approx. 96% was chosen since this most closely represented the specificity of the Bovigam IFN-γ test (de la Rua-Domenech et al., 2006). At this level of specificity, cut-offs of 1.204 ng/ml, 0.685 ng/ml and 1.638 ng/ml were obtained for ΔPPD, ESAT-6/CFP-10 and Rv3615c respectively.

Having defined the cut-offs for the specific test reagents, these were used to compare the sensitivity of the CXCL10 readout to that of IFN-γ in detecting BTB infected animals, which had been designated as infected by either a previous positive TB skin test reaction (group A) or a positive IGRA result (group C and D). To do so, CXCL10 and IFN-γ levels in samples from the same whole blood culture were analysed. Our initial investigations focused on the tuberculin test reagents (ΔPPD response), as these are used in the routine testing setting. As shown in Table 1, all 19 of the SICCT positive animals (group A) tested gave positive blood responses using IFN-γ as a readout (relative sensitivity of 100%, 95% CI 83–100%), while 17 were positive when using CXCL10

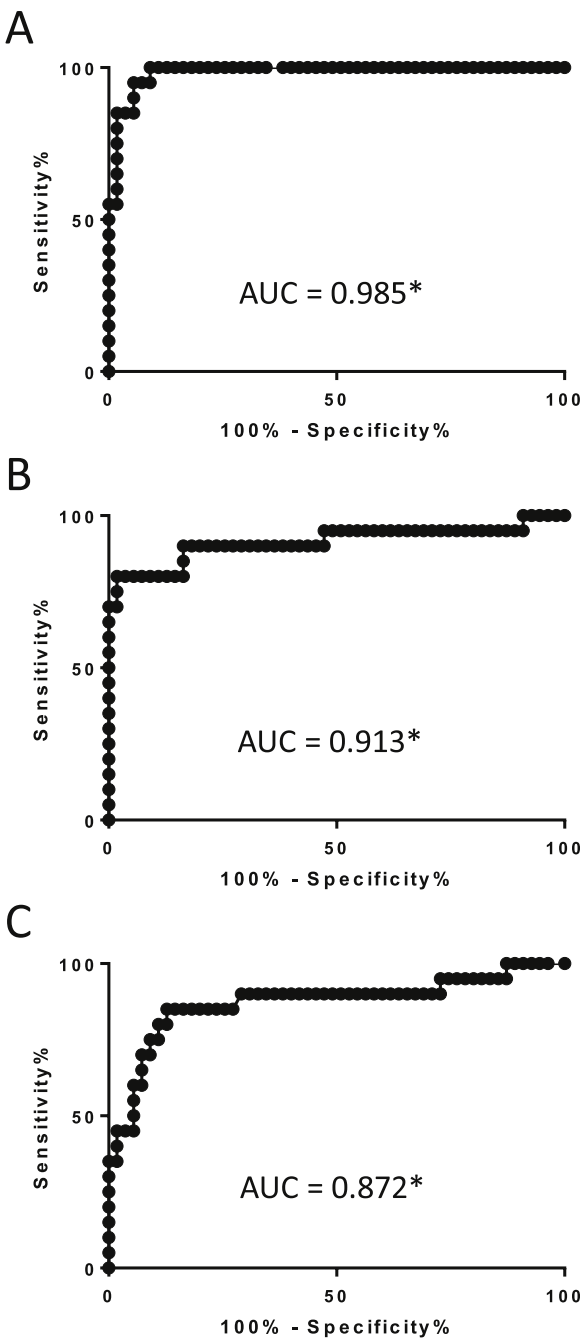

Fig. 1. Significant areas under the curve (AUC) for CXCL10 production in Receiver Operator Characteristic (ROC) curve analysis. ROC curves show the accuracies of (A) ΔPPD, (B) ESAT-6/CFP-10 and (C) Rv3615c induced CXCL10 production at distinguishing between TB reactor animals and uninfected controls. * $p < 0.0001$ (ROC curve analysis for AUC).

Table 1
Test positive outcomes using tuberculin (ΔPPD) induced IFN-γ and CXCL10 readouts.

	Test positive animals (% relative sensitivity)		
Readout	Group A (n = 19)	Group C (n = 22)	Group D (n = 71)
IFN-γ	19 (100%)	22 (100%)	71 (100%)
CXCL10	17 (89%)	9 (41%)*	31 (44%)*

* $p < 0.0001$, two-sided Fisher exact test (compared to IFN-γ).

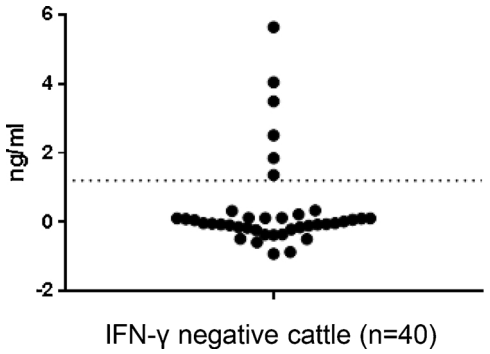

Fig. 2. Tuberculin-induced CXCL10 complements IFN-γ for the detection of BTB. CXCL10 levels were quantified in ΔPPD stimulated blood cultures from 40 tuberculin skin test (SICCT) positive animals (Group E) that tested negative using the IFN-γ readout. Circles represent individual samples and the horizontal line denotes the cut-off for positivity (set at 1.204 ng/ml).

(relative sensitivity of 89%, 95% CI 69–98%). As expected, all group C and group D animals tested positive in the blood test using IFN-γ, with relative sensitivities of 100% (95% CI 85–100% and 95% CI 95–100% respectively). In contrast, significantly smaller proportions of group C (9 out of 22) and group D (31 out of 71) animals tested positive using CXCL10, resulting in relative sensitivities of 41% (95% CI 23–61%) and 44% (95% CI 33–55%) respectively.

Although the results suggested CXCL10 could not substitute for IFN-γ as a test readout for the tuberculin whole blood assay, we next investigated whether it had potential as an additional readout to complement IFN-γ to maximise overall test sensitivity for the detection of *M. bovis* infection. To this end, we tested samples from 124 tuberculin skin test positive cattle in herds with current active outbreaks of BTB (group E). The samples were first screened with the standard IFN-γ assay, which revealed 84 test positive cattle. We then quantified CXCL10 levels in the remaining 40 samples that were not detected by the IFN-γ assay, which revealed a further 6 animals as test positive (Fig. 2). This improved the relative sensitivity of diagnosis in this setting from 68% (95% CI 59–75%) to 73% (95% CI 64–80%).

Having shown that CXCL10 has the potential to improve test sensitivity using conventional tuberculin reagents, we next investigated a similar role when specific antigens were used to stimulate whole blood. To this end, we used a cocktail of overlapping peptides covering the amino acid sequence of the immune-dominant antigens ESAT-6 and CFP-10. In addition, we investigated responses to a peptide cocktail covering the amino acid sequence of the antigen Rv3615c, as this has previously been shown to be useful in detecting infected animals that fail to respond to ESAT-6/CFP-10 (Sidders et al., 2008). When group A animals (SICCT positives) were considered, measurement of CXCL10 did not identify any additional test positives to the ESAT-6/CFP-10 peptide cocktail (Table 2). In contrast, a readout of IFN-γ and/or CXCL10 (compared to IFN-γ alone) identified an additional 2 animals testing positive to the Rv3615c peptide cocktail, resulting in an increase in relative sensitivity from 58% (95% CI 36–77%) to 68% (95% CI 46–85%). Significant improvement in relative test sensitivity was observed for both ESAT-6/CFP-10 and Rv3615c peptide cocktails in group C animals (ΔPPD IGRA positives). Six additional ESAT-6/CFP-10 test positive animals were identified using the IFN-γ and/or CXCL10 readout (compared to IFN-γ alone), resulting in an increase in relative sensitivity from 64% (95% CI 43–80%) to 91% (95% CI 72–98%). Only 1 group C animal tested positive to Rv3615c using IFN-γ alone (relative test sensitivity of 5%, 95% CI 0–22%), which increased to 6 when using the IFN-γ and/or CXCL10 readout (relative test sensitivity of 27%, 95% CI 13–48%). The ESAT-6/CFP-10 and Rv3615c peptide cocktails were designed for use in an IFN-γ blood DIVA test to identify BCG vaccinated animals that were not fully protected from infection with *M. bovis* (as determined by the presence of pathology at post mortem and/or culture of *M. bovis* from lymph node/lung tissue). From previous BCG vaccination / challenge experiments, we identified 18 confirmed infected animals that tested negative to these antigens when using IFN-γ as the test readout (group F animals). Of these, four animals were identified as ESAT-6/CFP-10 test positive when CXCL10 was used as an additional test readout (Table 2). Taken together, combining the data from all three groups highlighted the additional benefit of measuring CXCL10 to maximise the sensitivity of blood tests using specific antigens.

4. Discussion

Traditional IGRA assays for BTB are based on the detection and comparison of IFN-γ production to PPD-B and PPD-A. Using a similar approach, the CXCL10 readout failed to achieve a detection rate matching that for IFN-γ (Table 1), suggesting that a general replacement of an IFN-γ based readout by CXCL10 is not a viable option in blood-based assays for BTB. In agreement, a previous study in African buffaloes also demonstrated that CXCL10 based tests failed to identify all animals disclosed by a positive IFN-γ test (Goosen et al., 2015). Only one other reported study has investigated the diagnostic potential of CXCL10 for BTB in cattle (Parsons et al., 2016), where the ΔPPD CXCL10 readout identified all IGRA positive animals. In this study, the relative sensitivity of CXCL10 was evaluated using SICCT positive IGRA positive cattle, which would be equivalent to our group A animals where we also see the best relative sensitivity for CXCL10.

As shown in Table 1, CXCL10 showed the lowest potential for diagnosing infection in SICCT negative IGRA positive animals (groups C and D). Taking the results from several studies (Coad et al., 2008; Gormley et al., 2006; Lahuerta-Marin et al., 2015; Neill et al., 1994; Pollock et al., 2005) it is likely that these SICCT negative IGRA positive animals may be at an earlier stage of infection compared to SICCT positive IGRA positive animals (i.e. group A). Indeed, all group A animals showed evidence of disease pathology (visible lesioned; VL) at post mortem investigation. In contrast, only 30 of the 71 group D

Table 2
Test positive outcomes using specific antigen induced IFN-γ and CXCL10 readouts.

Readout	Test positive animals (% relative sensitivity)							
	Group A (n = 19)		Group C (n = 22)		Group F (n = 18)		Combined (n = 59)	
	E6/C10[a]	Rv3615c	E6/C10	Rv3615c	E6/C10	Rv3615c	E6/C10	Rv3615c
IFN-γ	15 (79%)	11 (58%)	14 (64%)	1 (5%)	0 (0%)	0 (0%)	29 (49%)	12 (20%)
CXCL10	15 (79%)	11 (58%)	18 (82%)	6 (27%)	4 (22%)	0 (0%)	37 (63%)	17 (29%)
Either	15 (79%)	13 (68%)	20[b] (91%)	6[b] (27%)	4 (22%)	0 (0%)	39[b] (66%)	19 (32%)

[a] E6/C10 refers to the ESAT-6/CFP-10 peptide cocktail.
[b] $p < 0.05$, one-sided Fisher exact test (compared to IFN-γ).

animals were VL (data not shown). When CXCL10 responses were compared between group D VL and NVL (non-visible lesioned) animals, a significantly greater proportion ($p < 0.05$, Fisher exact test) of test positive results were observed in VL (18 out of 30) compared to NVL (13 out of 41) animals (data not shown). Interestingly, these results are similar to those previously observed for IL-2 responses (Rhodes et al., 2014). Thus, although CXCL10 may not be sufficient to replace IFN-γ as a readout in blood assays for BTB, it may provide additional information with regards to disease pathology.

An alternative way in which CXCL10 could be used as a diagnostic tool is to maximise the potential of blood-based assays by detecting infected animals that tested negative using the standard IFN-γ readout. For tuberculin induced responses, we were limited to analysing animals in group E (SICCT positives), as all animals in groups A, C and D already gave positive ΔPPD IFN-γ responses. Of the 40 group E animals which tested negative for ΔPPD IFN-γ, 6 gave a positive response to CXCL10 (Fig. 2), demonstrating the potential of this biomarker to complement IFN-γ. This potential could be further enhanced if a cut-off for positivity predicted by the ROC curve analysis to give 100% sensitivity was used. In this case, a further 2 animals negative for ΔPPD IFN-γ gave CXCL10 responses (data not shown). Although altering the cut-off resulted in a decrease in overall CXCL10 test specificity (a decrease from 96% to 91%), it is possible that this may still remain high enough in situations where maximal sensitivity for detecting infection is required.

A real benefit for the use of additional biomarker readouts is in the context of assays that utilise specific mycobacterial antigens (ESAT-6, CFP-10 and Rv3615c), where detection of infection may not be as sensitive compared to tuberculin reagents (Sidders et al., 2008; Vordermeier et al., 2001). Although not improving detection of ESAT-6/CFP-10 responses, the additional measurement of CXCL10 did identify 2 extra test positives to the Rv3615c peptide cocktail in group A animals (Table 2). The measurement of CXCL10 appeared to play a greater diagnostic role in group C animals, where additional responders to both peptide cocktails were identified.

Cattle vaccination is being considered as part of BTB control strategies, and currently the only potentially available vaccine is BCG (bacille Calmette Guérin). As BCG vaccination is not fully protective in all cattle (Vordermeier et al., 2016), a diagnostic test will be required to identify BCG-vaccinated cattle that develop BTB upon exposure to *M. bovis*. Unfortunately, BCG vaccination compromises the specificity of current tuberculin-based diagnostic tests (Berggren, 1981; Buddle et al., 1995, 1999) and so a blood DIVA IFN-γ test using the ESAT-6/CFP-10 and Rv3615c peptide cocktails has been developed (Vordermeier et al., 2016). However, as this test is not 100% sensitive, there is potential for BCG vaccinated animals that are not fully protected from BTB to escape detection. We identified samples from 18 such animals from previous vaccination/challenge experiments (Dean et al., 2015; Jones et al., 2017) and demonstrated that additional measurement of CXCL10 could disclose a proportion of these animals as test positive (Table 2, group F), highlighting the potential for applying the CXCL10 assay in parallel with IFN-γ for the diagnosis of BTB in settings where tuberculin-based reagents cannot be used.

There are some technical and practical issues related to the CXCL10 assay that may still need to be addressed. As previously described (Parsons et al., 2016; Waters et al., 2012b), we also observed high levels of spontaneous CXCL10 production in the unstimulated control cultures from some animals (data not shown). Given that a comparative readout of CXCL10 production in peptide-stimulated cultures minus nil-antigen control cultures is used when evaluating responses to the ESAT-6/CFP-10 and Rv3615c peptide cocktails, this spontaneous release may limit the full diagnostic potential of CXCL10 in this setting. Furthermore, given that neither the IFN-γ nor the CXCL10 test readouts are set to 100% specificity, the use of a dual biomarker system may also compromise overall test specificity. When the production of both biomarkers were analysed in whole blood cultures from 50 control non-infected cattle (group B animals, data not shown), no positive IFN-γ responses were seen to any of the test reagents (ΔPPD, ESAT-6/CFP-10 and Rv3615c peptide cocktails), whereas 2 positive CXCL10 test outcomes were observed with each of the three different stimuli. Using the ROC curve analysis, it is possible to raise the cut-offs for these antigens to achieve 100% specificity in the CXCL10 assay. Although this resulted in overall fewer infected animals testing positive for CXCL10, added benefit in detecting responses to the peptide cocktails was still observed (Supplemental Table 1).

5. Conclusion

The results presented herein demonstrate that in particular settings measurement of CXCL10 has the potential to complement the current use of IFN-γ in blood assays to maximise the detection of BTB. This beneficial role is particularly evident when detecting responses to less immunodominant antigens (e.g. Rv3615c) or responses to specific antigen cocktails in animals at a potentially earlier stage of infection (e.g. group C).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.01.007.

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