Investigating the biomarker potential of host proteins and development of lateral flow assays to detect *Mycobacterium bovis* infection

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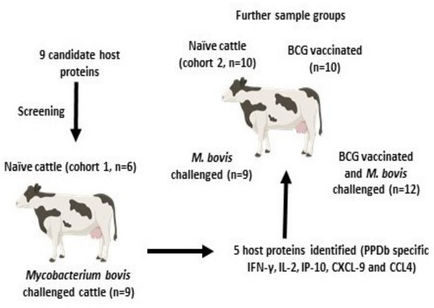
1. Introduction

- *Mycobacterium bovis* (*M. bovis*), a globally prevalent pathogen, causes zoonotic tuberculosis (zTB) in humans and bovine tuberculosis (bTB) in cattle; with significant public, animal welfare and economic impact. Tuberculin Skin Test (TST) and Interferon Gamma Release Assay (IGRA) are the commercially available diagnostic tests for cattle. Problems with implementing test-and-slaughter measures in Low and Middle Income Countries (LMICs) in combination with under-performance of TST and IGRA in field situations establish a clear need to develop improved diagnostics.
- Due to early onset of cell-mediated immune responses, a number of cytokine and chemokine markers have been described with significant gene expression differences in experimentally challenged animals compared to naïve controls. Comprehensive evaluation of these candidate host proteins are lacking; fuelling the rationale for this study.

Hypothesis and Approach

- "According to bTb infection status, there is a differential protein expression of cytokines and chemokines i.e. potential biomarkers in bovine tuberculin (PPDb) stimulated whole blood supernatants (WBS) and serum samples".
- Based on reported gene expression differences and availability of anti-bovine ELISA reagents; nine chemokine and cytokine proteins were screened in PPDb stimulated WBS and serum samples obtained from *M. bovis* challenged and bTB naïve animals by performing Enzyme linked immunosorbent assays (ELISAs). Six of the most promising proteins were used to develop user-friendly Lateral Flow Assays (LFAs) based on Upconverting reporter Particles (UCP) technology.

2. ELISA study design and methods



- Blood samples were collected into heparin or for serum. Whole blood was stimulated with PPDb or medium for 24h and supernatants were removed and stored frozen prior to analysis of cytokines and chemokines by ELISA.
- Animals were naïve, BCG vaccinated and infected (challenged) with *M. bovis* strain AF2122/97 with and without prior BCG vaccination
- Data are shown as PPDb-specific pg/ml and statistical comparisons were performed using Kruskal-Wallis Test with Dunn's multiple comparison post-test.

3. Cytokine and chemokine levels in naïve and *M. bovis* challenged animals

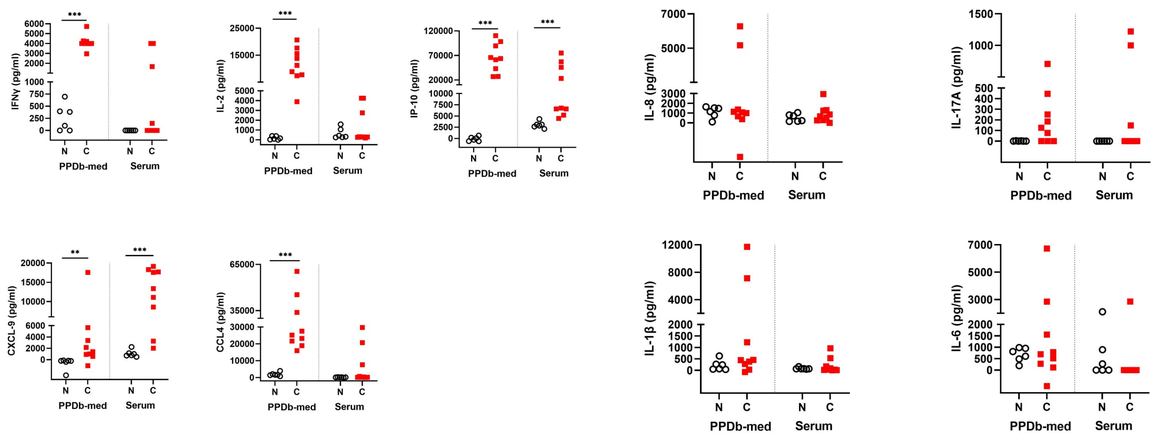


Figure 1: Cytokine and chemokine levels in PPDb stimulated whole blood supernatants and serum samples from naïve (N, n=6, cohort 1) and *M. bovis* challenged (C, n=9) animals.

- **IFNγ, IL-2, IP-10, CXCL9 and CCL4** were found to be significantly higher in PPDb stimulated WBS of challenged animals (left) while for **IL-1β, IL-6, IL-8 and IL-17A** (right), no significant differences were detected. Serum concentrations were found to be generally low with only CXCL9 and IP-10 detected with significant differential potential.
- The five proteins significant in initial assessment (IFNγ, IL-2, IP-10, CXCL9 and CCL4) were evaluated in additional samples.

4. PPDb specific protein levels can differentiate *M. bovis* infection and BCG vaccination from naïve animals

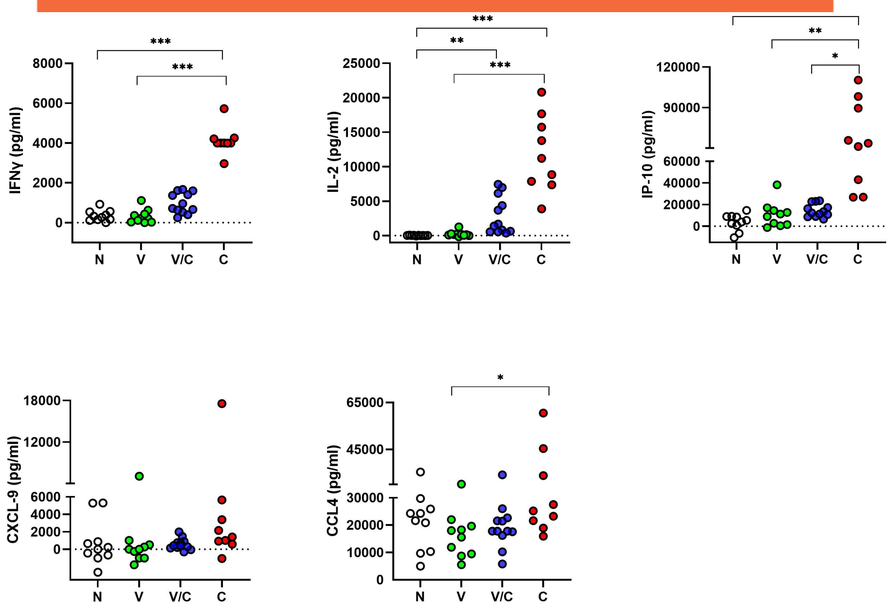


Figure 2: Protein levels of IFNγ, IL-2, IP-10, CXCL-9 and CCL4 in stimulated whole blood supernatants. PPDb specific expression is shown. N: Naïve Animals (cohort 2, n=10, empty circles), V: BCG vaccinated animals (n=10, green circles), V/C: BCG vaccinated and *M. bovis* challenged (n=12, blue circles) and C: *M. bovis* challenged only (n=9, red circles). *, p < 0.05; **, p<0.01; ***, p<0.001.

- PPDb specific IL-2 and IP-10; like IFNγ; can identify *M. bovis* infection. These with CCL4 also have a DIVA potential i.e. **D**ifferentiating *M. bovis* **I**nfected animals from BCG **V**accinated **A**nimals.

5. Upconverting Particles (UCP) based Lateral Flow Assays (LFAs) – Workflow and Development

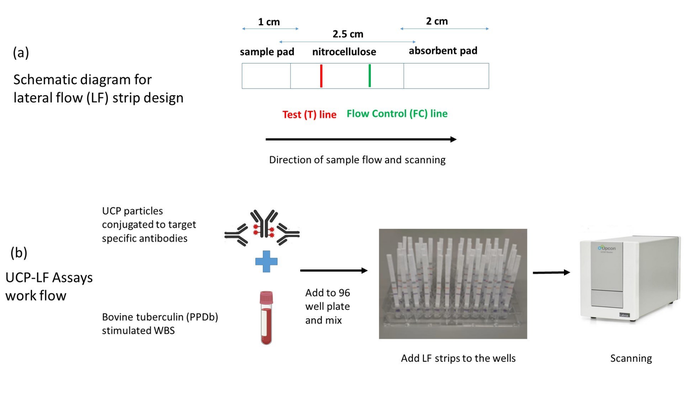


FIGURE 3: Schematic diagram of lateral flow (LF) strip design (a) and workflow of UCP-LFAs (b).

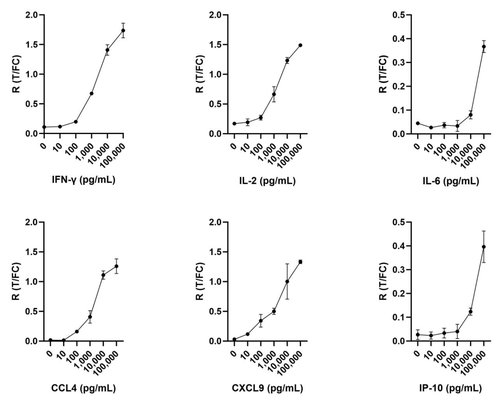


Figure 4: Respective recombinant cytokines were spiked in assay buffer and analysed using UCP-LFAs. Shown are the ratios (R) obtained by dividing the signal at the respective test (T) lines by the signal at the flow control (FC) lines. Mean values with error bars (±1 SD) are shown.

- UCP-LFAs can detect respective recombinant bovine proteins.

6. Differences between animal groups based on ratios determined using UCP-LFAs

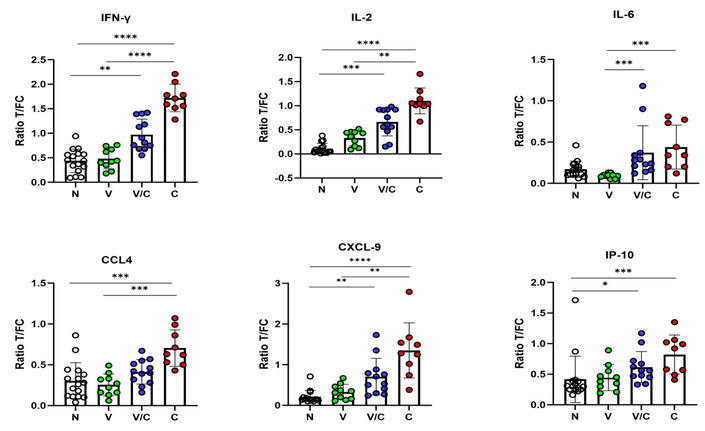


FIGURE 5: IFN-γ, IL-2, IL-6, CCL4, CXCL9, and IP-10 levels in PPDb-stimulated whole blood supernatants were measured by UCP-LFAs. The ratios among groups were compared using the Kruskal–Wallis test with Dunn's multiple comparison post-test. N: Naive Animals (n = 16, empty circles); V: BCG-vaccinated animals (n = 10, green circles); V/C: BCG-vaccinated and *Mycobacterium bovis* challenged (n = 12, blue circles); C: *M. bovis* challenged only (n = 9, red circles). The bars of scatter dot plots show mean values, and error bars show ±1 SD. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

- Ratios determined by UCP-LFAs can differentiate animal groups based on their *M. bovis* infection and BCG vaccination status.

7. NUM Scoring and Correlation Analysis

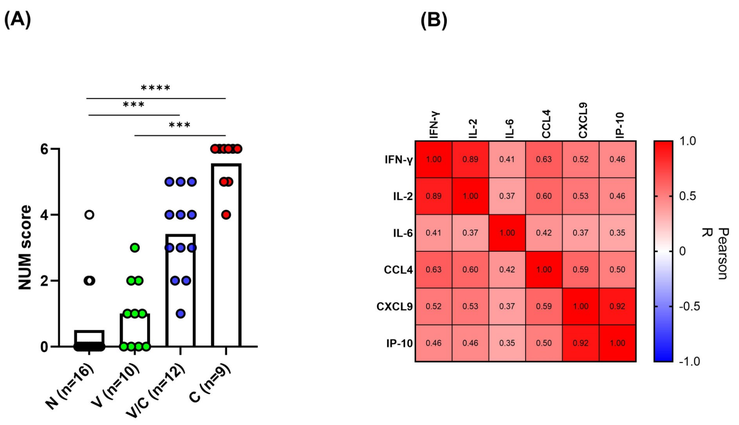


FIGURE 6: NUM Score and correlation analysis. A NUM score was calculated based on IFN-γ, IL-2, IL-6, CCL4, CXCL9, and IP-10 ratios in PPDb-stimulated whole blood supernatants for naïve (N; n = 16), BCG-vaccinated (V; n = 10), and *M. bovis* challenged animals with (V/C; n = 12) or without prior BCG vaccination (C; n = 9). The NUM score (y-axis) combines the results of six proteins, indicating the number of proteins with levels above a threshold based on the maximal Youden's index for each marker [Table 1(a)]. Group differences were determined using the Kruskal-Wallis test; the statistical significance level used was ***p < 0.001; ****p < 0.0001 (A). Heatmap showing Pearson correlation among the ratios of the evaluated host proteins. The colour corresponds to the Pearson R value as indicated in each square (B).

- Individual outcomes can be combined (by NUM Scores for example) and correlation analysis can help in selection of markers to find a "multibiomarker signature" that can have enhanced discrimination potential.

8. Limitations

- Preliminary data with limited sample size; samples from one breed and time-point with PPDb as stimulating antigen
- Optimization of assays

9. Ongoing/Future work

- Validation of results on additional kinetic cohorts of *M. bovis* experimentally challenged animals, field TB reactors, animals with confounding infections using samples stimulated using *M. bovis* specific antigens
- Development of mAbs for improvement in IP-10 assay
- Testing for additional promising biomarkers
- Developing a user-friendly, multiplex UCP-LFA

Take Home Message

- These are our initial findings towards the eventual aim of developing a multibiomarker UCP-LFA test. Simultaneous quantitative detection of multiple analytes on a single user-friendly format can be an attractive "One Health" solution for controlling of bTB and zTB; particularly for resource constrained settings.

References

- **Khalid, Hamza**, Anouk van Hooij, Timothy K. Connelley, Annemieke Geluk, and Jayne C. Hope. 2022. "Protein Levels of Pro-Inflammatory Cytokines and Chemokines as Biomarkers of *Mycobacterium bovis* Infection and BCG Vaccination in Cattle" *Pathogens* 11, no. 7: 738. https://doi.org/10.3390/pathogens11070738
- **Hamza Khalid**, Louise Pierneef, Anouk van Hooij, Zijie Zhou, Danielle de Jong , Elisa M.T.K. Fat, Timothy K. Connelley, Jayne C. Hope, Paul L.A.M. Corstjens, and Annemieke Geluk. 2023. " Development of lateral flow assays to detect host proteins in cattle for improved diagnosis of bovine tuberculosis" *Front. Vet. Sci.* 10:1193332. https://doi.org/10.3389/fvets.2023.1193332