Indian Journal of Experimental Biology
Vol. 59, June 2021, pp. 415-420

Interferon gamma and IP 10 mRNA quantitative real time PCR in whole blood culture of guinea pig and cattle to multi-antigen recombinant protein cocktail and PPD of *Mycobacterium bovis* (3/86Rv)

Sangram Ramane & Rishendra Verma*

Mycobacteria Laboratory, Division of Bacteriology and Mycology, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly - 243 122, Uttar Pradesh, India

Received 15 December 2019; revised 28 February 2021

In vivo tuberculin skin test and *in vitro* IFNγ assays are the most explored diagnostics for *Mycobacterium bovis* infection (Bovine Tuberculosis) in animals. However, there are other potential biomarkers like IP10 which may allow diagnosis of tuberculosis. In this study, IFNγ and IP10 mRNA responses in guinea pig and cattle were determined by quantitative RT-PCR in whole blood samples stimulated PPDs and recombinant protein cocktail containing eight purified rCFP2, rESAT6, rCFP10, rMTC28, rMPB53, rMPB63, rMPB64 and rMPB83 proteins. Blood samples were obtained from *M. bovis* sensitised guinea pigs and tuberculin test positive cattle. Both guinea pigs and cattle blood cultures produced a significant increase in mRNA level of IFNγ and IP10 when stimulated with protein cocktail as compared to that of bovine PPD. Upon different antigenic stimulations, IP10 mRNA responses were followed the similar kinetics to those of IFNγ with high correlation (r = 0.97 for guinea pig and 0.96 for cattle).

Keywords: Bovine tuberculosis, Cattle, Livestock, Piggery

Tuberculosis (TB) is a worldwide disease of animals and human caused by bacteria belonging to *Mycobacterium tuberculosis* complex (MTBC). *M. bovis*, a member of MTBC, with a broadest host range is considered as the principal etiologic agent of TB in animals[1]. *M. bovis* infection elicits a strong T lymphocyte mediated immune (CMI) response in the host[2]. World Organization for Animal Health (previously OIE) recommends tuberculin skin test (TST) as a prescribed test for bovine tuberculosis (BTB) diagnosis for the international trade while interferon gamma (IFNγ) release assay as an alternative test[3], both of these are CMI response based tests. The IFNγ release assay is an *in vitro* test, where amount of IFNγ protein in the PPD stimulated blood plasma is quantified by either enzyme-linked immunosorbent assay (ELISA)[4] or enzyme-linked immunospot assay (ELISPOT)[5].

In recent years, reverse transcription qPCR (RT-qPCR) has facilitated accurate quantification of mRNA transcript levels in samples taken from a wide range of tissue types. Cytokine mRNA expression has also been measured far earlier before protein synthesis begun and correlates well with cytokine protein level[6]. Diagnostic test based on IFNγ is considered to be more sensitive than TST[7] but sometimes indiscriminate results are also obtained[8] which can be overcome using new biomarkers such as IFNγ inducible protein 10 (IP10)[9]. The paper reports IFNγ and IP10 mRNA responses in guinea pig and cattle whole blood samples stimulated with multi-antigen recombinant protein cocktail formulation, PPD of *M. bovis* strain (3/86Rv) and avian PPD.

Materials and Methods

Mycobacterial strains and purified protein derivatives (PPDs)

M. bovis 3/86 strain, a field strain isolated from cattle lymph node in 1986 and routinely maintained on Lowenstain-Jensen media, was obtained from Mycobacteria Laboratory, Division of Bacteriology and Mycology, IVRI, Izatnagar. Bovine and avian PPD (PPDb and PPDa) prepared as per the procedure described earlier by Garg and Verma[10].

Recombinant mycobacterial proteins

Study targeted rCFP2, rESAT6, rCFP10, rMTC28, rMPB53, rMPB63, rMPB64 and rMPB83 proteins of *M. bovis* strain 3/86 already expressed and purified from *E. coli* BL21 (DE3-pLysS) using pET32b

*Correspondence
Phone: +91 9359117376
E-Mail: rishendra_verma@yahoo.com

expression vector in Mycobacteria Laboratory, IVRI[11-14]. The Limulus Amebocyte Lysate PYROGENT™ kit (Lonza, Walkersville, USA) was used as an *in vitro* end-product endotoxin level detection test prior to cocktail preparation as per the manufacturer's guidelines. Multi-antigen protein cocktail consisted 0.5 µg of each rCFP2, rESAT6, rCFP10, rMTC28, rMPB53, rMPB63, rMPB64 and rMPB83 protein.

Experimental animals and sensitization

The experiment had approval of the Institute Animal Ethics Clearance (IAEC) (F.No.1-53/2012-13-J.D. (Res). Six clinically healthy adult Dunkin-Hartley (200-300 g) guinea pigs (*Cavia porcellus*) of either sex obtained from the Laboratory Animal Resource Section (IVRI, Izatnagar) were housed under standard laboratory conditions of housing, food and water. All guinea pigs were sensitized by deep intramuscular injection with 0.5 mL of a suspension in liquid paraffin containing 4 mg/mL of heat-killed *M. bovis* bacilli[12,13]. Four cattle from an organized farm at Bareilly, India were also included who had history of tuberculosis and those were positive to the TST.

Blood culture

Two separate blood culture experiments were carried out for guinea pig and cattle blood[15]. First experiment was performed on blood samples from *M. bovis* sensitized guinea pigs while second experiment was performed on blood samples collected from tuberculin test positive cattle. The whole heparinized blood collected was mixed with equal proportion of RPMI 1640 medium (Sigma, USA) and 1.0 mL of the mixture was added to each well of the 24-well culture plate in aseptic conditions. The medium was supplemented with antibiotics (penicillin 50 U/mL and streptomycin 50 µg/mL) to reduce the risk of contamination. One of five antigens consisting of PPDb (30 µg/mL), PPDa (30 µg/mL), concavillin A (5 µg/mL), multi-antigen cocktail (4 µg/mL *i.e.* 0.5 µg of each rCFP2, rESAT6, rCFP10, rMTC28, rMPB53, rMPB63, rMPB64 and rMPB83 proteins) and PBS (pH 7.4, control) was added in culture plates in triplicate under aseptic conditions. The culture plates were gently swirled on a smooth flat surface and incubated statically at 37°C in 5% humidified CO_2 tension for 6 h.

Total RNA isolation and cDNA synthesis

After 6 h of incubation, the cultured cells were pelleted by centrifugation at 2000 G for 10 min. In a fresh 1.5 mL microcentrifuge tube, 200 µL sedimented cells of blood culture was mixed with 1 mL of the RiboZol reagent (Amresco, USA) and RNA was extracted as per the manufacturer's instructions with final elution volume of 20 µL DEPC treated water. To remove any possible contamination by genomic DNA, the samples were submitted to digestion with DNase (RNase-free DNase Set, Qiagen). The isolated RNA was assessed for quality and quantity using Nanodrop Spectrophotometer (ND-1000, Thermo Fisher Scientific, USA) and stored at −80°C untill further use. Reverse transcription of total RNA (2 µg) was carried out using a RevertAid cDNA synthesis kit (Thermo Scientific, USA) as per the manufacturer's recommendations employing 200 U of RevertAid reverse transcriptase enzyme and oligodT (0.5 µg) during synthesis. The cDNA product was stored at −20°C.

Quantitative PCR

The primers for guinea pig and cattle IFNγ, IP10 and endogenous housekeeping gene β-actin used in present study have been enumerated in Table 1[13,15,16]. The qPCR was performed on cDNAs isolated from six guinea pig sensitized with heat killed *M. bovis* 3/86 and four tuberculin test positive cattle blood samples stimulated with PPDb, PPDa, ConA, cocktail and PBS in MX3000P Real Time PCR System (Stratagene, La Jolla, USA) using 3 µL of cDNA, 10 µL 2X SsoFast EVA Green Master Mix (BioRad, USA), 10 pM of forward and reverse primers and the volume was made up to 20 µL using nuclease free water. Cycling parameters for the Real Time PCR was initial denaturation for 10 min, followed by 40 cycles at 95°C for 30 s, 56°C (guinea pig) / 53°C (cattle) for 30 s and 72°C for 30 sec. Melting curve analysis were performed for each sample to verify the specificity of each product. The real-time data obtained was analyzed by MxPro™ QPCR Software version 4.10

Table 1 — Primer sequences used in present study

Gene	Primer	Sequence (5' to 3')
Bovine IFNγ[13]	Forward	GAA TTG GAA AGA TGA AAG TGA C
	Reverse	CAG AGC TGC CAT TCA AGA AC
Bovine β-actin[15]	Forward	CAA TGA AGA TCA AGA TCA TCG C
	Reverse	GTG TAA CGC AGC TAA CAG TC
Bovine IP10[This study]	Forward	AAG TCA TTC CTG CAA GTC AAT CCT
	Reverse	TTG ATG GTC TTA GAT TCT GGA TTC AG
Guinea pig IFNγ[13]	Forward	GAC TGT CCA AAA TAG CAT GAA C
	Reverse	TCA TTG ACC GAA ATT TGA ATC
Guinea pig β-actin[16]	Forward	CTT TGC TGC GTT ACA CCC
	Reverse	GTC ACC TTC ACC GTT CCA
Guinea pig IP10[This study]	Forward	AGA ACA ATA CGC TGC ACC
	Reverse	TGA TCT CAA CTC GTG GGC

(Stratagene, La Jolla, USA). Intra-assay and inter-assay variability were determined for each target gene and each sample was run in triplicate. The cycle threshold (Ct) values and amplification plot for guinea pig IFNγ and bovine IFNγ were acquired by using the 'EvaGreen with Dissociation Curve' method of the real time machine.

Statistical analysis

Data recorded was analyzed by PROC GLM in SAS version 9.3 (SAS Institute Inc., Cary, North Carolina, USA) software. The change in IFNγ and IP10 mRNA expression level in the antigen-stimulated blood cultures relative to that in the control were calculated using the ΔΔCt method[17]. The statistical significance of differences in IFNγ and IP10 mRNA expressions of the examined factors was assessed by one-way ANOVA. Differences were considered significant if $P < 0.05$. Correlation between IFNγ and IP10 expression level was calculated using the CORREL function or the Analysis Toolpak add-in in MS Excel.

Results and Discussion

Serum tests suffer from poor sensitivity and recently WHO has disagreed on antibody based testing of tuberculosis[18]. The production of IFNγ protein by immune cells can be measured using ELISA in Bovigam assays and found to be comparably sensitive for BTB diagnostics[19]. Relative quantification of IFNγ mRNA may strongly indicate *M. bovis* infection, IFNγ being a critical cytokine of host defences against *M. bovis* infection[15]. Identification of additional biomarkers like IP10 supplementing IFNγ could further enhance diagnostic performance of *in vitro* assays. Serum levels of IP10 have been evaluated as biomarkers for diagnosis of tuberculosis[9] but only few reports regarding IP10 mRNA quantification by qPCR. Our aim was to authenticate the multi-antigen cocktail containing eight recombinant proteins *viz.*, rCFP2, rESAT6, rCFP10, rMTC28, rMPB53, rMPB63, rMPB64 and rMPB83, as alternative to PPD by relative quantification of IFNγ and IP10 cytokine expression using real time PCR. Here, two independent experiments were carried out by using blood from experimentally *M. bovis* sensitized guinea pigs and blood from tuberculin test positive cattle as described earlier[15]. A fixed incubation time point of 6 h was selected for analysis of blood cultures in response to PPDb, PPDa, conA and antigenic cocktail for IFNγ and IP10 gene expression, as studies had shown that IFNγ mRNA level is found high from 4 hrs to 6 hrs in blood from tuberculosis infected animals[20].

First experiment follows relative expression of IFNγ and IP10 mRNA from *M. bovis* sensitized guinea pigs using non-stimulated blood culture as control. A significantly increase in mRNA expression of IFNγ gene was observed after stimulation of blood with antigens (Fig. 1A). The expression of IFNγ mRNA was found 20.91±3.41, 10.73±1.73, 44.06±4.12 and 32.23±3.63 fold increase in PPDb, PPDa, ConA and cocktail stimulated blood samples, respectively as compared to non-stimulated control. The IFNγ mRNA expression level of cocktail stimulated group was found significantly up regulated ($P < 0.01$) when compared with PPDb group. Similarly, the relative expression of IP10 mRNA after 6 h of stimulation was found up regulated. The fold increase of IP10 mRNA expression was found 9.30±0.90 and 14.35±1.44 for PPDb and cocktail stimulated guinea pig blood samples, respectively as compared to non-stimulated

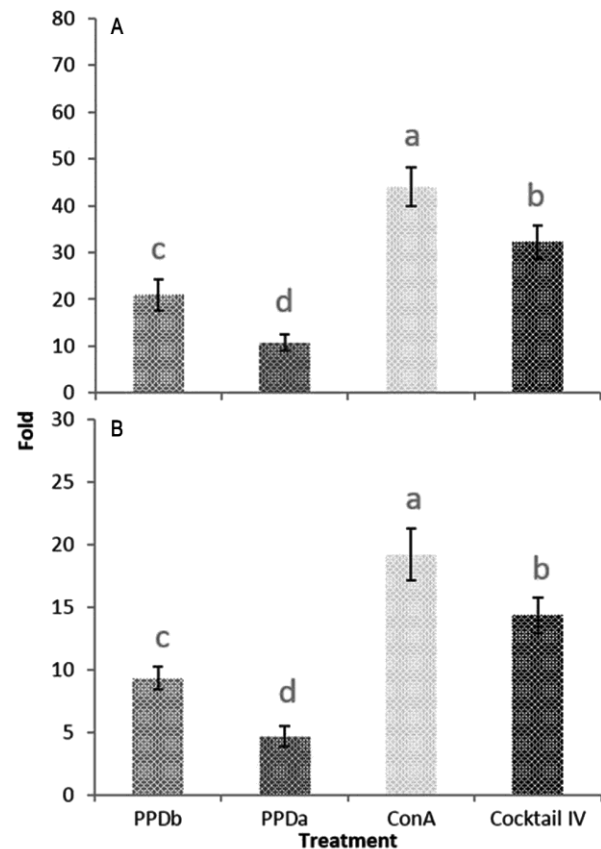

Fig. 1 — Relative expression of (A) IFNγ mRNA; and (B) IP10 mRNA from blood of experimentally *M. bovis* sensitized guinea pigs after antigenic stimulation

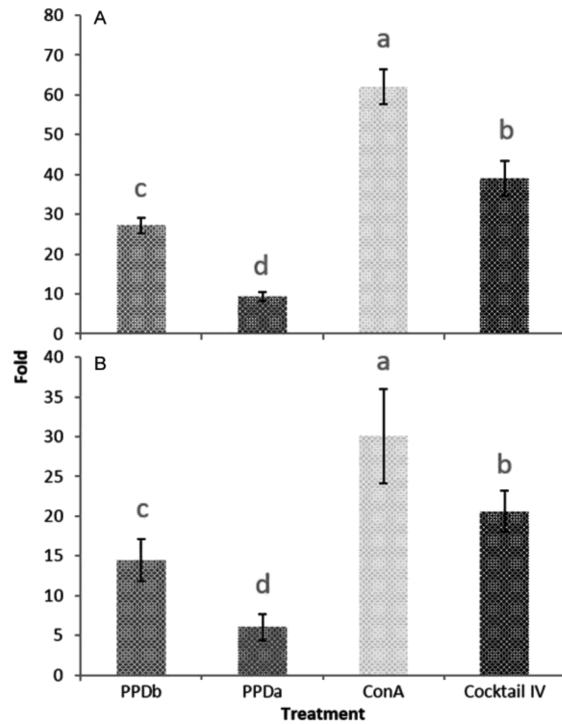

Fig. 2 — Relative expression of (A) IFNγ mRNA; and (B) IP10 mRNA from blood of tuberculin test positive cattle after antigenic stimulation

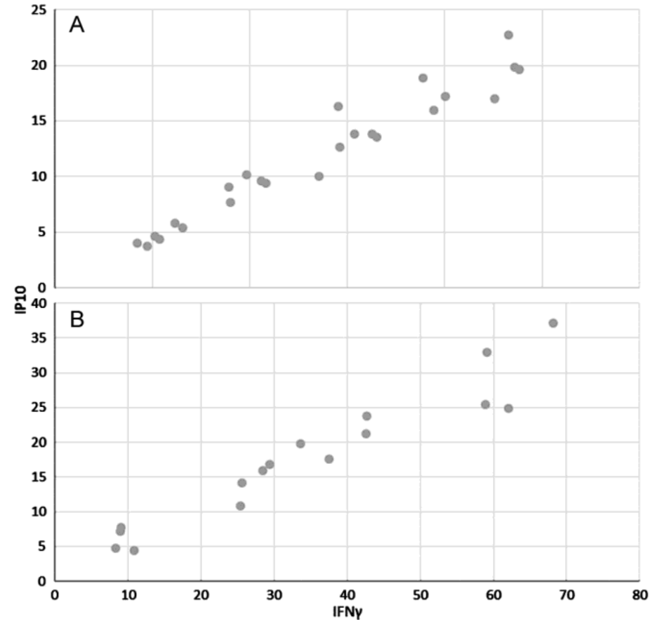

Fig. 3 — Correlation between (A) guinea pig IFNγ and IP10 mRNA response (r = 0.97); and (B) bovine IFNγ and IP10 mRNA response (r = 0.96)

control (Fig. 1B). The IP10 mRNA expression level due to cocktail was significantly higher (P <0.01) than PPDb stimulation. In second experiment, relative expression of IFNγ mRNA after stimulation of the whole blood from tuberculin positive cattle blood was significantly up regulated as compared to non-stimulated control. The fold increase of IFNγ mRNA expression was found 27.20±2.00 and 39.08±4.37 for PPDb and cocktail stimulated cattle blood samples, respectively as compared to healthy control (Fig. 2A). The IFNγ mRNA expression level due to cocktail was significantly higher (P <0.01) than PPDb stimulation. Similarly, the relative expression of IP10 mRNA after 6 h of stimulation was found up regulated. The fold increase of IP10 mRNA expression was found 14.43±2.65 and 20.62±2.57 for PPDb and cocktail stimulated cattle blood samples, respectively as compared to non-stimulated control (Fig. 2B). The IP10 mRNA expression level due to cocktail was significantly higher (P <0.01) than PPDb stimulation.

PBMCs were tested with complex mycobacterial antigens and pools of synthetic peptides. The results showed that MPT83 was among the strongest Th1 cell antigens of *M. tuberculosis*[21] and MPT63 induced moderate Th1 cell reactivity[22]. CFP2, a low molecular weight secreted protein from *M. tuberculosis*, also found to induce IFNγ *in vitro* in significantly high level tuberculous peripheral blood mononuclear cells[23]. MPB64 was also found to be recognised more frequently by TB patients and had produced significant IFNγ response in whole blood assay[24]. All of these ESAT6, CFP10, CFP2, MPB83 and MPB63 with MPB64 were the components of our multi-antigen cocktail which support the data obtained in our study related to IFNγ mRNA response. Like IFNγ, IP10 response to mitogen and antigen is inherently continuous[25]. A lower IP10 mRNA expression response was observed to mycobacterial antigens after sensitization in guinea pig and cattle, a better reason for this will be a higher concentration of IP10 in blood in non-stimulated whole blood cultures than those in antigen stimulated cultures[26].

Correlation between IP10 and IFNγ mRNA responses for guinea pig and cattle was calculated. Comparison included all the antigenic stimulation and found a strong correlation. Correlation coefficient (r) was calculated, it was 0.97 and 0.96 for guinea pig and cattle, respectively (Fig. 3 A and B). In both animal species, upon different antigenic stimulation, IP10 mRNA responses followed the similar kinetics to those of IFNγ. IP10 and IFNγ mRNA expression responses were found to be highly correlated in our study with a correlation coefficient for guinea pig was

0.97 and for cattle it was 0.96. Ting and co-workers also showed that levels of PPDb stimulated IP-10 (mRNA and protein), and ESAT6-CFP10 induced IP-10 (mRNA and protein) were significantly higher in cattle naturally or experimentally infected with *M. bovis* than in those that were uninfected[27]. Blauenfeld *et al*.[28] compared diagnostic potential of a molecular IP-10 release assay and they showed high technical performance of a molecular assay detecting IP-10 mRNA expression in infection with *M. tuberculosis*.

Conclusion

The present study showed that IFNγ and IP-10 mRNA responses had significant increased as compared to stimulation by PPDb in *M. bovis* sensitized guinea pig and tuberculin positive cattle quantified by RT-PCR in whole blood samples stimulated with cocktail of recombinant purified proteins. IP10 mRNA response followed similar kinetics to those IFNγ with high correlation. The results of our study in guinea pigs and cattle using multi-antigen cocktail of eight recombinant proteins may foster detection of TB in cattle by the detection of IFNγ and IP-10, and suggest that it could be considered as major diagnostic cocktail of recombinant purified proteins for the detection of in *M. bovis* infection in animals.

Conflicts of interest

Authors declare no competing interests.

References

1 O'Reilly, LM & Daborn CJ, The epidemiology of *Mycobacterium bovis* infections in animals and man: a review. *Tuber Lung Dis*, 76 (1995) 11.

2 de la Rua-Domenech R, Goodchild AT, Vordermeier HM, Hewinson RG, Christiansen KH & Clifton-Hadley RS, Ante mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. *Res Vet Sci*, 81 (2006) 190.

3 OIE, Office International des Epizooties (OIE) terrestrial manual: Chapter 2.4.7. Bovine Tuberculosis, World Health Organization for Animal Health, Paris, France. (2014) 686.

4 Wood PR & Jones SL, BOVIGAM: An i*n vitro* cellular diagnostic test for bovine tuberculosis. *Tuberculosis*, 81 (2001) 147.

5 Lalvani A, Pathan AA, Durkan H, Wilkinson KA, Whelan A, Deeks JJ, Reece WH, Latif M, Pasvol G & Hill AV, Enhanced contact tracing and spatial tracking of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells. *Lancet*, 357(9273) (2001) 20.

6 Turtle L, Kemp T, Davies GR, Squire SB, Beeching NJ & Beadsworth MB, In routine UK hospital practice T-SPOT.TB is useful in some patients with a modest pre-test probability of active tuberculosis. *Eur J Intern Med*, 23 (2012) 363.

7 Sellam J, Hamdi H, Roy C, Baron G, Lemann M, Puechal X, Breban, M, Berenbaum F, Humbert M, Weldingh K, Salmon D, Ravaud P, Emilie D & Mariette X, Comparison of *in vitro* specific blood tests with tuberculin skin test for diagnosis of latent tuberculosis before anti-TNF therapy. *Ann Rheum Dis*, 66 (2007) 1610.

8 Matulis G, Juni P, Villiger PM & Gadola SD, Detection of latent tuberculosis in immunosuppressed patients with autoimmune diseases: performance of a *Mycobacterium tuberculosis* antigen-specific interferon gamma assay. *Ann Rheum Dis*, 67 (2008) 84.

9 Roos EO, Olea-Popelka F, Buss PE, de Klerk-Lorist L-M, Cooper DV, Warren RM, van Helden PD, Parsons SCD, & Miller MA, IP-10: A potential biomarker for detection of *Mycobacterium bovis* infection in warthogs (*Phacochoerus africanus*). *Vet Immunol Immunopathol*, 201 (2018) 43.

10 Garg A & Verma R, Quality control and diagnostic efficiency of bovine PPD (tuberculin) from indigenous *Mycobacterium bovis*. *Indian J Anim Sci*, 78 (2008) 908.

11 Ramane SP, Verma R, Mondal T & Upmanyu V, Tuberculin response in guinea pigs with recombinant proteins cocktail prepared from Indian strain of *Mycobacterium bovis* (3/86 Rv). *Indian J Exp Biol*, 56 (2018) 638.

12 Ramane SP, Evaluation of recombinant proteins cocktail (MPB64, MPB83 and ESAT6) as diagnostic candidate for bovine tuberculosis. PhD Thesis submitted to Indian Veterinary Research Institute, Izatnagar, India, 2014.

13 Mondal T, Verma R, Ramane SP & Chakravarti S, Recombinant CFP-10 as antigen for diagnosis and quantification of IFN-γ expression by real-time PCR in guinea pigs sensitized with *Mycobacterium bovis*. *Indian J Exp Biol*, 56 (2018) 875.

14 Samal A, Verma R, Upmanyu V & Verma H, Molecular cloning and expression of MPT63 gene of *Mycobacterium tuberculosis*. *Indian J Anim Sci*, 85 (2015) 947.

15 Gan W, Zhou X, Yang H, Chen H, Qiao J, Khan SH, Yang L, Yin X & Zhao D, Development of a test for bovine tuberculosis in cattle based on measurement of gamma interferon mRNA by real-time PCR. *Vet Rec*, 173 (5) 117.

16 Lu L, Du X, She W, Zhang X, Wu Q, Yuan W & Chan F, Relationship of glucocorticoid receptor expression in peripheral mononuclear cells and cochlea of guinea pigs and effects of dexamethasone administration. PLoS One 8, e56323.

17 Livak KJ & Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 25 (2001) 402.

18 Lyashchenko K, Whelan AO, Greenwald R, Pollock JM, Andersen P, Hewinson RG & Vordermeier HM, Association of tuberculin-boosted antibody responses with pathology and cell-mediated immunity in cattle vaccinated with *Mycobacterium bovis* BCG and infected with *M. bovis*. *Infect Immun*, 72 (2004) 2462.

19 Gormley E, Doyle M B, Fitzsimons T, McGill K & Collins JD, Diagnosis of *Mycobacterium bovis* infection in cattle by use of the gamma-interferon (Bovigam) assay. *Vet Microbiol*, 112 (2006) 171.

20 Kim S, Kim YK, Lee H, Cho JE, Kim HY, Uh Y, Kim YM, Kim H, Cho SN, Jeon BY & Lee H, Interferon gamma mRNA quantitative real-time polymerase chain reaction for the diagnosis of latent tuberculosis: a novel interferon gamma release assay. *Diagn Microbiol Infect Dis*, 75 (2013) 68.

21 Mustafa AS, Comparative evaluation of MPT83 (Rv2873) for T helper-1 cell reactivity and identification of HLA-promiscuous peptides in *Mycobacterium bovis* BCG-vaccinated healthy subjects. *Clin Vaccine Immunol*, 18 (2011) 1752.

22 Mustafa AS, Th1 cell reactivity and HLA-DR binding prediction for promiscuous recognition of MPT63 (Rv1926c), a major secreted protein of *Mycobacterium tuberculosis*. *Scand J Immunol*, 69 (2009) 213.

23 Lee JS, Son JW, Jung SB, Kwon YM., Yang CS, Oh JH, Song CH, Kim HJ, Park JK, Paik T H & Jo EK, *Ex-vivo* responses for interferon-gamma and proinflammatory cytokine secretion to low-molecular-weight antigen MTB12 of *Mycobacterium tuberculosis* during human tuberculosis. *Scand J Immunol*, 64 (2006) 145.

24 Roche PW, Winter N, Triccas JA, Feng CG & Britton WJ, Expression of *Mycobacterium tuberculosis* MPT64 in recombinant *M. smegmatis*: Purification, immunogenicity and application to skin tests for tuberculosis. *Clin Exp Immunol*, 103 (1996) 226.

25 Ruhwald M, Petersen J, Kofoed K, Nakaoka H, Cuevas LE, Lawson L, Squire SB, Eugen-Olsen J & Ravn P, Improving T-cell assays for the diagnosis of latent TB infection: potential of a diagnostic test based on IP-10. *PLoS ONE*, 3 (2008) e2858.

26 Whittaker E, Gordon A & Kampmann B, Is IP-10 a better biomarker for active and latent tuberculosis in children than IFNgamma? *PLoS ONE*, 3 (2008) e3901.

27 Ting X, Xintao G, Hongjun Y, Pingjun L, Qianqian L, Shaohua H, Xiukun S, Xiaoyu G, Weifeng Y, Hongfei Z, Jiabo D & Hong J, Limitations of using IL-17A and IFN-γ-Induced Protein 10 to detect bovine tuberculosis. *Front Vet Sci*, 5 (2018) 28.

28 Blauenfeldt T, Villar-Hernández R, García-García E, Latorre I, Holm LL, Muriel-Moreno B, De Souza-Galvão ML, Millet JP, Sabriá F, Sánchez-Montalva A, Ruiz-Manzano J, Pilarte J, Jiménez MA, Centeno C, Torres C, Molina-Pinargote I, González-Díaz YD, Santiago J, Cantos A, Prat C, Andersen P, Domínguez J & Ruhwald M, Diagnostic accuracy of interferon gamma-induced protein 10 mRNA release assay for tuberculosis. *J Clin Microbiol*, 58(10) (2020) e00848-20.