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# Cytokine gene expression of IFN-γ, TNF-α and IL-4 in cattle and buffaloes suspected of bovine tuberculosis

**Pallvi Slathia, Deepti Narang, Mudit Chandra, Sikh Tejinder Singh and Maroudam Veeraswamy**

**Abstract**
A total of 20 blood samples were collected from bTB reactor animals (n=10) and bTB non-reactor animals (n=10) collected from cattle and buffaloes to study the cytokine gene expression of IFN- γ, TNF-α and IL-4 in both the bTB reactor group of animals and the non-reactor group of animals. Real time PCR was used to study the gene expression in *in vitro* DST (MPB83,70) stimulated PBMCs. IL-4 gene expression was seen only in 4 animals. Ct value of IL-4 ranged from 32 to 34 (in bTB reactor animals) and >36 (in non-reactor animals). When RQ of these samples was calculated it showed an upregulation in one animal (RQ 1.380) while it was down regulated in three animals (RQ 0.0268, 0.128 and 0.752) when compared with bTB negative animal. TNF-α gene expression was seen only in 3 animals. Ct value of TNF-α ranged from 30 to 33 (in bTB reactor animals) and >34 (in non-reactor animals). Upregulation was observed in only one sample (RQ 2.702) and down regulation was observed in two samples (0.266 and 0.213) when compared to non-reactor group of animals based on their RQ value. IFN-γ expression gene expression was seen in 5 animals. Ct value of IFN-γ ranged from 30 to 35 (in bTB reactor animals) and >37 (in non-reactor animals). IFN-γ expression was found to be upregulated in 4 bTB reactor animals (RQ 6.294, 1.142, 3.92 and 1.034) and downregulated in one animal (RQ 0.22) based on RQ value.

**Keywords:** Real time PCR, DST (MPB83, 70), bTB reactor, bTB non-reactor IFN-γ, TNF-α and IL-4

## Introduction
*Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex (MTC), is the cause of the chronic disease known as bovine tuberculosis (bTB), which affects cattle. It immediately affects production, which has a substantial economic impact on cattle farming, and has the potential to spread zoonotic diseases, raising concerns for public health (Renwick *et al*., 2007) [7]. While most high-income countries have strict test and cull strategies in place to effectively control bTB, the disease is still endemic in the majority of low- and middle-income nations, where national control programmes have not yet been put into place for socioeconomic reasons. As a result, the disease continues to significantly reduce animal productivity as well as cause human morbidity and mortality (Pollock *et al*., 2014) [5].
The current method for diagnosis of bTB in animals measures cell-mediated immune response following an intradermal skin test with the poorly defined and highly variable tuberculin skin test (TST) antigen. Comparative genomic, transcriptomic and proteomic analyses have identified several *M. bovis* antigens having DIVA capability. These antigens include the highly immunogenic proteins ESAT-6, CFP-10 and Rv3615c that are present in *M. bovis* but either absent or not immunogenic in all BCG vaccine strains. These when used in combination as defined antigens have shown considerable promise in detecting *M. bovis*-infected animals and in differentiating them from those vaccinated with BCG in both skin test and laboratory assays and are suitable test for routine surveillance and diagnosis of bTB-infected animals, thereby paving the way for the development of a much needed "defined antigen skin test" (DST) with DIVA capability (Srinivasan *et al*., 2019) [9].

## Materials and Methods
For cytokine gene expression studies, 10 bTB reactors (CID test positive) from either cattle or buffaloes and equal number of bTB negative animals (CID test negative) were selected from organized dairy farms and blood samples (n=20) was collected from these bTB positive reactor and non-reactor animals. Blood samples were collected and mononuclear cells were isolated by density gradient centrifugation method using HiSep™ LSM 1084 (Hi Media). Blood

samples (n=20) were collected from cattle and buffaloes in heparinized vials under sterile conditions and the samples were diluted using sterile PBS in 1:2 ratio. In a sterile 50 ml centrifuge tube, HiSep™ LSM 1084 (Hi Media), 15 ml was taken and equal amount of diluted blood was slowly layered over it. The tubes were then centrifuged for 30 minutes at 400 x g. After centrifugation, 4 layers were formed and the mononuclear cell layer or opaque interface (PBMCs layer), was thoroughly aspirated using a sterile pipette and transferred to a clean 15 ml or 50 ml centrifuge tube. The PBMCs were then washed with sterile PBS by centrifuging at 400 x g for 10 minutes. The washing step was repeated 3 times. The final washing was done using RPMI 1640 medium containing 10% FCS. After this, the PBMCs were suspended in 1 ml of RPMI 1640 medium containing 10% FCS (Boyam, 1968 and Kishore *et al*., 2014). After this, 250 µl of this cell suspension was transferred in each 46 well flat bottom plate. Then, 25 µl of defined skin antigen, MPB83,70 (0.5 mg) was added into it and the plate was then incubated for 4 hours in a humidified atmosphere at 5% $CO_2$ at 37 ºC. After incubation, the plate was centrifuged at 800 x g for 10 minutes for harvesting cells in RPMI 1640 medium (lysate). The cells were then processed using ReliaPrep™ RNA Cell Miniprep system (Promega) kit for RNA extraction and cDNA was synthesised from RNA using ImProm-II™ Reverse Transcription System.

**Cytokine gene expression of IFN-γ, TNF-α and IL-4**

TaqMan Real-Time PCR assay was done for gene expression of cytokines TNF-α, IFN-γ (Xin *et al*., 2018) [13] and IL-4 (Widdison *et al*., 2006) [12] using specific primers and probes (Table: 1). Probes specific for *M. bovis* sequence DNA were obtained from (IDT ltd.) and master mix from Applied Biosystems (ABI) respectively.

**Table 1:** List of primers and probes

| Cytokine | Primer | Probe |
|---|---|---|
| Il-4 | FP: 5' -GCCACACGTGCTTGAACAAA-3'<br>RP: 5' -TCTTGCTTGCCAAGCTGTTG-3' | FAM 5TCCTGGGCGGACTTGACAGGAATC3'BkFQ |
| TNF-α | FP- 5′-AGAAATTAGGGATGTAGGGAAGTGA-3′<br>RP: 5′-CTTGTGGACCCCAGGGAGTT-3′ | FAM AGCCCACGTTGTAGCCGACATCAACTCC BkFQ |
| IFN-γ | FP: 5′-TGGATATCATCAAGCAAGACATGTT-3′<br>RP: 5′-ACGTCATTCATCACTTTCATGAGTTC-3′ | FAM CAGATCATCCACCGGAATTTGAATCAGC BkFQ |

**TaqMan real-time assay protocol**

TaqMan real-time assay was performed with the Applied Biosystems (ABI) Step one plus real-time PCR. The protocol for the reaction mixture prepared for the assay is given in Table 2 and the cycling conditions are shown in Table 3. Analysis of the TaqMan assay was done as per ABI step one plus real-time PCR software.

**Table 2:** TaqMan real-time PCR reaction mixture for the gene expression of cytokines

| S. No. | PCR Components | Volume used per reaction |
|---|---|---|
| 1. | TaqMan Master Mix (2X) | 10 µl |
| 2. | Primer-Probe mix (20X) | 1 µl |
| 3. | Nuclease free water | 7 µl |
| 4. | DNA template | 2 µl |
| | Total Volume | 20 µl |

**Table 3:** Cycling conditions for TaqMan qRT-PCR assay

| Stage | Step | Temperature (°C) | Duration | No. of Cycles |
|---|---|---|---|---|
| I | Initial denaturation | 95 | 10 min | 1 |
| II | Denaturation | 95 | 15 seconds | 40 |
| III | Annealing and extension | 60 | 1 min | |
| IV | Final extension | 72 | 30 seconds | 1 |

**Results and Discussion**

Immunity against mycobacterial infection is mostly based on a cell-mediated response involving macrophages, dendritic cells and an adaptive T cell response. Cytokines regulate the actions of these cells and the close proximity of cells within the granuloma is thought to enhance immunological responses (Flynn and Chan, 2001) [3]. Cytokines have a wide range of functions; some are pro-inflammatory such as IFN-γ, activating immune cells to kill mycobacteria and inducing a type I immune response, while others, such as interleukin IL-4 and IL-10 are anti-inflammatory, suppressing the proinflammatory immune response to prevent damage and can be determined using techniques such as Real time PCR (Saunders *et al*., 2000) [8].

A number of intriguing M. bovis antigens with DIVA capability have been discovered using comparative genomic, transcriptomic, and proteomic investigations, including the highly immunogenic proteins ESAT-6, CFP-10, and Rv3615c that are present in M. bovis but either lacking or not immunogenic in all BCG vaccination strains (Vordermeier *et al*., 2016) [11]. These proteins have showed great promise when used in conjunction as specified antigens for identifying M. bovis-infected animals and distinguishing them from those who have received the BCG vaccination in both skin tests and laboratory experiments (Srinivasan *et al*., 2019) [9].

The cytokine gene expression was studied by stimulating bovine PBMCs using DST (MPB83,70). *In vitro* DST-stimulated bovine PBMCs were used to estimate the cytokine gene expression profile of IFN- γ, TNF- α and IL-4 both in the bTB reactor group of animals and the non-reactor group of animals. For this study, a total of 20 blood samples were collected from bTB reactors (n=10) and bTB non-reactor animals (n=10). The samples were subjected to TaqMan Expression assays to quantify the gene expression of cytokines using Beta-actin as an endogenous control. The non-reactor animals were used as reference sample having RQ value of 1.

**Gene expression of IL-4**

In present study, out of 10 bTB reactor animals, gene expression of IL-4 was seen only in 4 animals. Ct value of IL-4 ranged from 32 to 34 (in bTB reactor animals) and >36 (in non bTB reactor animals). When RQ of these samples was calculated it showed an upregulation in one animal (RQ 1.380) while it was down regulated in three animals (RQ 0.0268, 0.128 and 0.752) when compared with bTB negative animal. Amplification plot of IL-4 expression profile in TB

reactor animals and RQ vs sample is given in Table 4 and Figure 1. According to a study conducted by Widdison *et al.* (2006) [12], the suppressed levels of IL-4 in infected animals compared to IFN-γ indicates a specificity in the suppression, not a generalized consequence of infection and necrosis due to the developing chronic immune response.

**Table 4:** Gene expression of IL-4 in bTB reactor animals

| Sample (bTB reactor) | Ct value | RQ |
|---|---|---|
| Sample 1 | 34.410 | 0.0268 |
| Sample 4 | 33.953 | 0.128 |
| Sample 5 | 32.760 | 0.752 |
| Sample 6 | 32.687 | 1.380 |

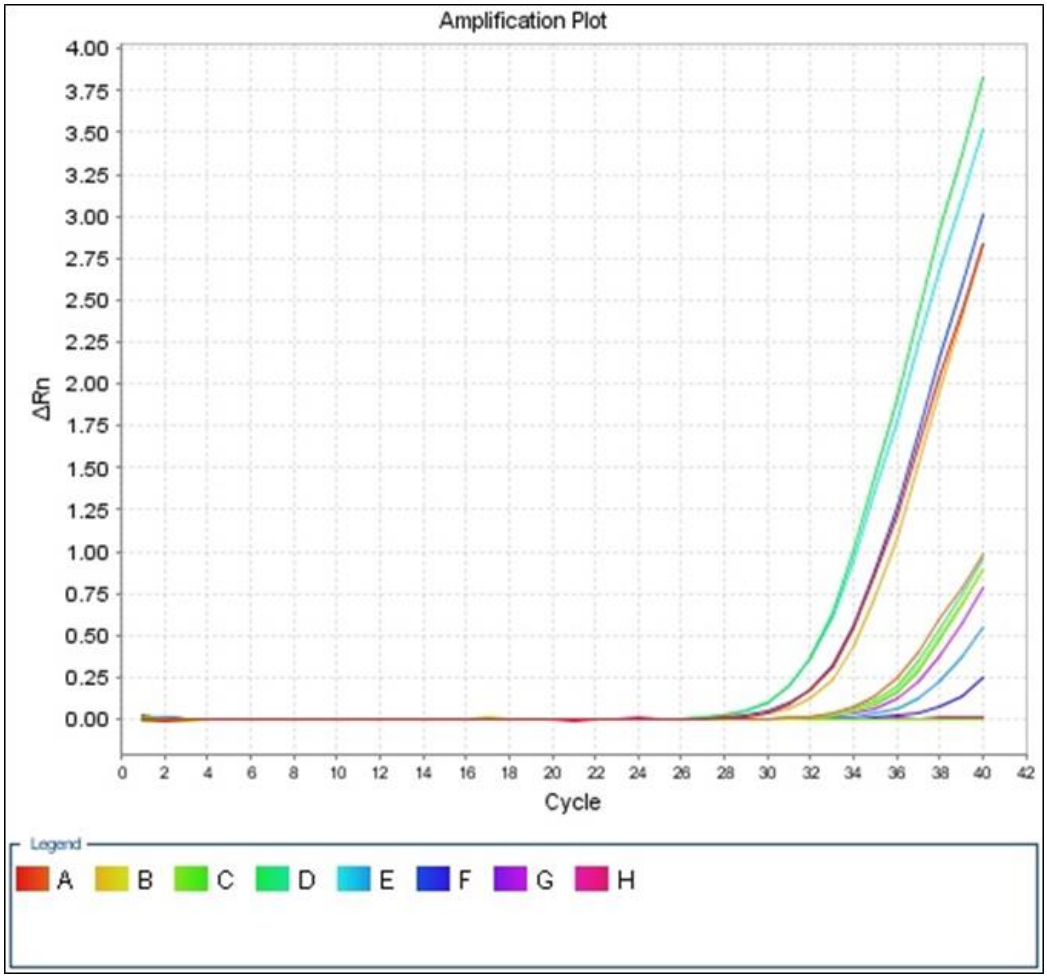


**Fig 1:** Amplification plot of IL-4 expression profile in bTB reactor animals

**Gene expression of TNF-α**

Amplification plot of TNF-α expression profile, Ct value and RQ vs sample graph are given in Table 5 and Figure 2. Upregulation was observed in only one sample (RQ 2.702) and down regulation was observed in two samples (0.266 and 0.213) when compared to non-reactor group of animals based on their RQ value. Ct value of TNF-α ranged from 30 to 33 (in bTB reactor animals) and >34 (in non-reactor animals).

**Table 5:** Gene expression TNF-α of in bTB reactor animals

| Sample (bTB reactors) | Ct value | RQ |
|---|---|---|
| Sample 1 | 30.01 | 2.702 |
| Sample 3 | 33.87 | 0.266 |
| Sample 5 | 33.670 | 0.213 |

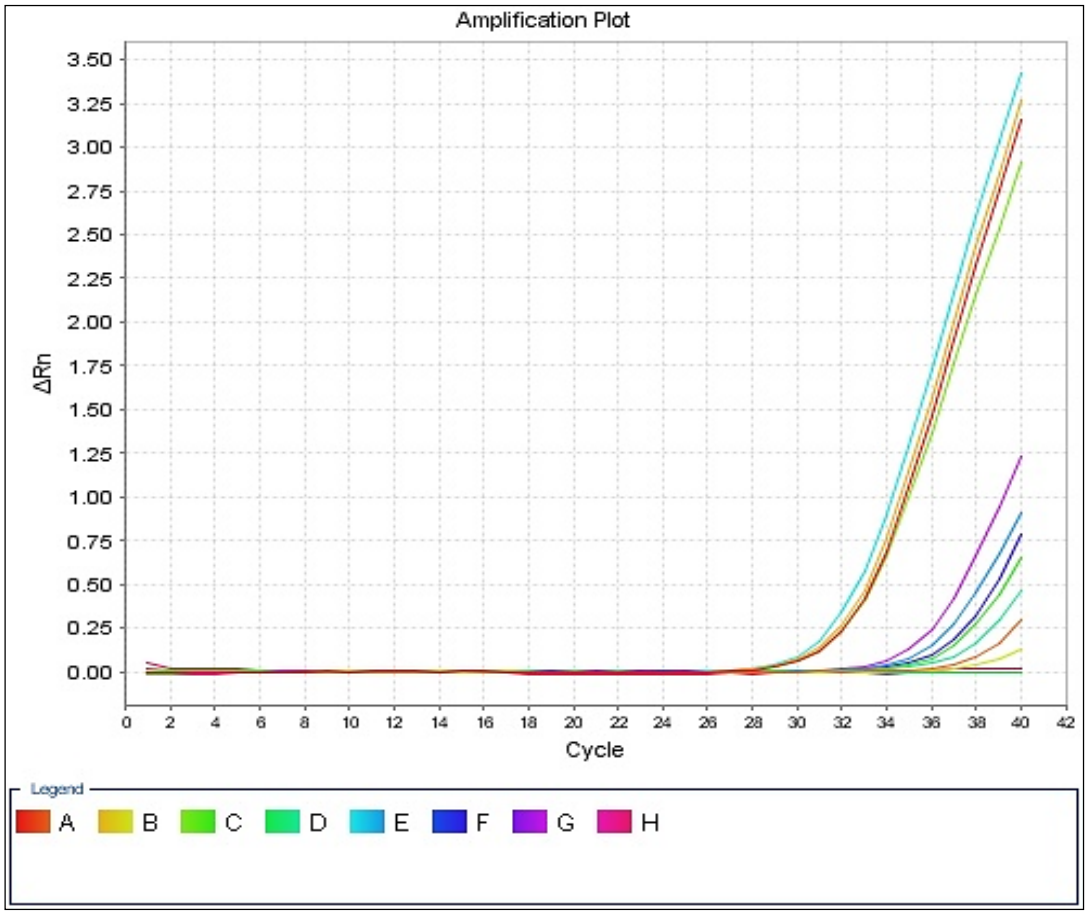


**Fig 2:** Amplification plot of TNF-α expression profile in bTB reactor animals

**Gene expression of IFN-γ**

Amplification plot of IFN-γ expression profile and RQ vs sample are given in Table 6 and Figure 3. Ct value of IFN-γ ranged from 30 to 35 (in bTB reactor animals) and >37 (in non-reactor animals). IFN-γ expression was found to be upregulated in 4 bTB reactor animals (RQ 6.294, 1.142, 3.92 and 1.034) and downregulated in one animal (RQ 0.22) based on RQ value. IFN-γ is a key cytokine produced in *M. bovis* infected cattle. In a similar study conducted by Xin *et al.* (2018) [13], PPD-B and CE-stimulated PBMCs from *M. bovis*-infected cattle had considerably higher levels of IFN-γ mRNA than those from uninfected cattle.

Arriaga *et al.* (2021) [1] reported a higher level of also examined gene expression of IFN-*γ*, IL-4 using Bovine PPD stimulated PBMCs from *M. bovis*-infected cattle. In a study conducted by Thacker *et al.* (2007) [10] the cytokine gene expression in 10 Holstein calves given intratonsillar inoculation of *M. bovis* were evaluated using purified protein derivative of *M. bovis* (PPD), 10 kD culture filtrate antigen and 10 kD culture filtrate antigen recombinant protein (rESAT6:CFP10) as stimulating antigens and found that IFN-γ, TNF-α, iNOS and IL-4 expression were increased in response to *M. bovis* infection. The response of IFN-*γ* and IP10 mRNA in blood samples was studied by Ramane and Verma, (2021) [6] using real time PCR. samples were stimulated with PPD and recombinant protein cocktail including eight purified proteins including rMPB83, rCFP2, rESAT6, rCFP10, rMPB53, rMPB63, rMPB64 and rMTC28 and concluded that stimulation with a protein cocktail produced a significant rise in IFN-*γ* levels when compared to bovine PPD.

**Table 6:** IFN-γ gene expression profile with RQ values

| Sample (bTB reactors) | Ct value | RQ |
|---|---|---|
| Sample 1 | 30.75 | 6.294 |
| Sample 2 | 31.11 | 1.412 |
| Sample 3 | 32.258 | 3.92 |
| Sample 4 | 35.994 | 0.22 |
| Sample 8 | 30.886 | 1.034 |

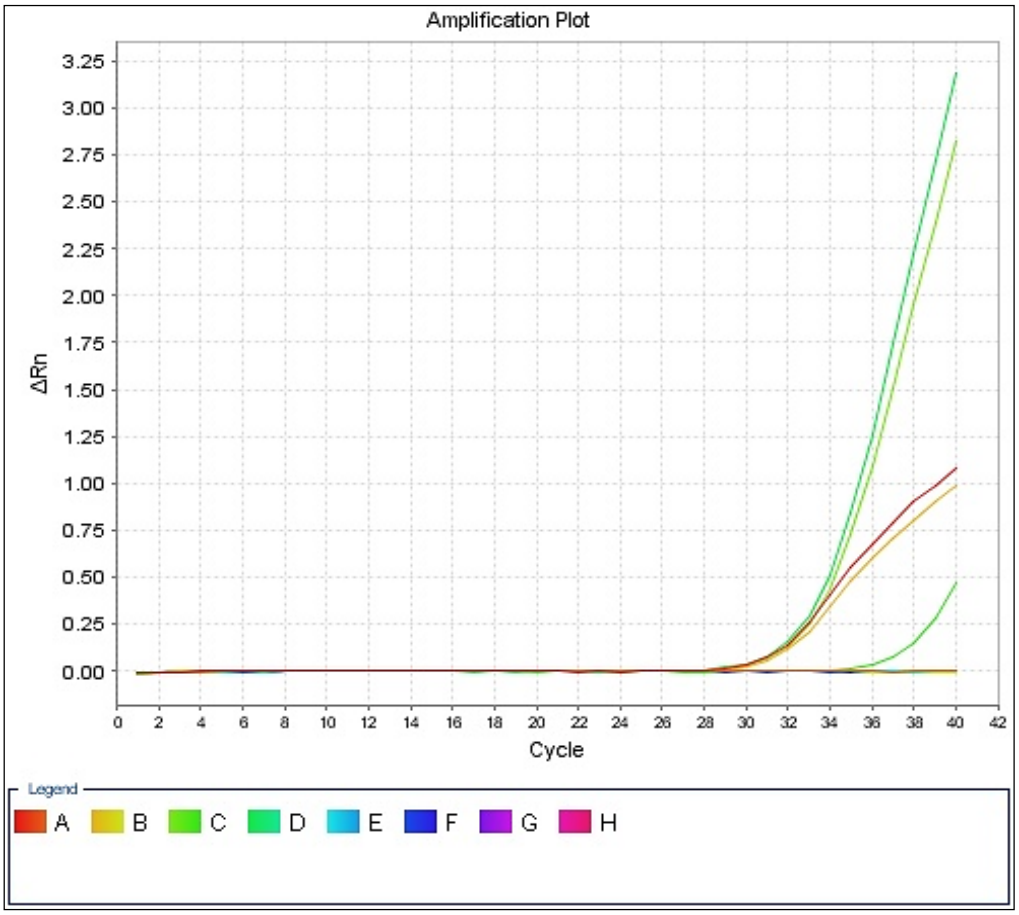


**Fig 3:** Amplification plot of IFN-γ expression profile in bTB reactor animals

## Conclusion

The current investigation showed that whole blood samples stimulated with DST (MPB83,70), significantly increased the IFN-$\gamma$, TNF-α and IL-4 responses in *M. bovis* sensitised and tuberculin-positive cattle.

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**Conflict of Interest:** Authors have no conflict of interest in this study.

**Authors contribution:** Pallvi Slathia: Collection of relevant literature, sample collection, laboratory work and manuscript preparation. Deepti Narang: Conceptualization of study design and interpretation. Mudit Chandra: Sample collection and interpretation. Sikh Tejinder Singh: Sample Collection.

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