Acta Scientific Veterinary Sciences (ISSN: 2582-3183)

Volume 7 Issue 9 September 2025

Research Article

# Cytokine Gene Expression of IFN-γ and TNF-α in Animals Infected with Johnes Disease

**Sanmeet Kour[1], Deepti Narang[2]*, Mudit Chandra[2], Sikh Tejinder Singh[2], Ashwani Kumar Sharma[3], Ramneek[4] and Kuldip Gupta[3]**

*[1]Ph.d Research Scholar, Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Punjab, India*

*[2]Principal Scientist, Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Punjab, India*

*[3]Professor, Department of Veterinary Medicine, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Punjab, India*

*[4]Professor, Animal Disease Research Centre, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Punjab, India*

***Corresponding Author:** Deepti Narang, Principal Scientist, Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Punjab, India.

**Received:** July 18, 2025

**Published:** August 26, 2025

## Abstract

*Mycobacterium avium* subspecies paratuberculosis (MAP) causes Johne's disease, a chronic wasting condition that affects ruminants and causes intestinal inflammation and intermittent diarrhea. The immune response to *Mycobacterium avium* subspecies *paratuberculosis* is mostly dependent on cellular immunity, and Th1 cell-produced cytokines are particularly crucial. Early infections with *Mycobacterium avium* subspecies paratuberculosis (MAP) appear to be frequently accompanied by the production of pro-inflammatory cytokines like IFN- γ and TNF-α by Th1-type T cells. The present study was conducted to analyze the cytokine gene expression of JD positive animals which were detected positive by IS900 -PCR and were compared to five healthy animals. The gene expression of IFN- γ was found to be more upregulated in animals than TNF- α as increased production of IFN- γ is characteristic of the infection caused by *Mycobacterium* as it is considered critical for control of mycobacterial infections.

**Keywords**: MAP; Cytokines; TNF- α, IFN- γ; IS900 PCR

## Introduction

Johne's disease caused by *Mycobacterium avium* subspecies *paratuberculosis* is responsible for huge economic losses as it causes chronic granulomatous infectious enteritis in wide range of animals which in turn decreases the milk production, lowers the fertility rate and increases the chances of premature culling. Cattle, buffaloes and sheep are most commonly affected by MAP, which damages the ileum and disrupts the intestinal lining, resulting in intermittent diarrhoea and decreasing the capacity of afflicted animals to absorb nutrients. With the progression of the disease the number of villus structures appears to diminish as inflammation increases, and areas of MAP infection are often observed to have significant numbers of infiltrating macrophages [7]. Earlier researches have described several characteristics of MAP infection and possible immune responses to MAP using both peripheral blood mononuclear cells and tissues infected with it.

The Th1 response i.e. the initial host response to MAP infections is characterized by the generation of IFN-γ. As the infection pro-

**Citation:** Deepti Narang*., et al.* "Cytokine Gene Expression of IFN-γ and TNF-α in Animals Infected with Johnes Disease". *Acta Scientific Veterinary Sciences* 7.9 (2025): 23-29.

gresses, MAP can continue to exist in the host by tricking the immune system by influencing the immune system of the host. Th1 response generates pro-inflammatory cytokines like IFN- γ, IL-6, IL-1, and IL-2 [2]. A change from a cell-mediated (Th1) response to a non-protective antibody-mediated (Th2) response marks the switching from a subclinical, or asymptomatic, to a clinical stage of infection. The relationship between macrophages, the preferred host cell for intracellular MAP infection and T cells has received considerable attention. It has been proposed that immune cells from sub clinically infected cows with MAP initially respond to MAP antigens with a classical Th1-like immune response, including production of IFN- γ and that clinical stages of Johne's disease (JD) appear to coincide with a shift to a classical, humoral Th2-like immune response [3]. TNF-α does not seem to be as significant with MAP in cattle, despite the possibility that it could be a key mediator of inflammation in MAP-infected tissues, similar to some tissues affected by Crohn's disease in humans [5].

## Material Methods

### Collection of samples

Table 1 enlists the blood samples (n = 20) collected from cattle and buffaloes which were detected positive by IS900 PCR for JD and five samples from healthy animals were collected from organized dairy farms.

| S. No. | Lab No. | Age | Species | Breed |
|---|---|---|---|---|
| 1 | 1 | 4 yrs. | Cattle | Crossbred |
| 2 | 2 | 3 yrs. | Cattle | Indigenous |
| 3 | 3 | 5 yrs. | Cattle | Crossbreed |
| 4 | 4 | 4.5 yrs. | Cattle | Sahiwal |
| 5 | 5 | 3 yrs. | Cattle | Crossbreed |
| 6 | 6 | 4.5 yrs. | Cattle | Sahiwal |
| 7 | 7 | 4 yrs. | Cattle | Indigenous |
| 8 | 8 | 4yrs. | Cattle | Sahiwal |
| 9 | 9 | 3 yrs. | Buffalo | Crossbred |
| 10 | 10 | 4 yrs | Buffalo | Sahiwal |
| 11 | 11 | 4.5 yrs | Cattle | Sahiwal |
| 12 | 12 | 3.5 yrs | Cattle | Crossbred |
| 13 | 13 | 3 yrs | Cattle | Sahiwal |
| 14 | 14 | 2.5 yrs | Cattle | Indigenous |
| 15 | 15 | 3 yrs | Cattle | Sahiwal |
| 16 | 16 | 4 yrs | Cattle | Crossbred |
| 17 | 17 | 4 yrs | Cattle | Crossbred |
| 18 | 18 | 3 yrs | Cattle | Sahiwal |
| 19 | 19 | 2.5 yrs | Cattle | Sahiwal |
| 20 | 20 | 3 yrs | Cattle | Indigenous |
| 21 | 21 | 4 yrs | Cattle | Sahiwal |
| 22 | 22 | 3.5 yrs | Cattle | Sahiwal |
| 23 | 23 | 3 yrs | Cattle | Indigenous |
| 24 | 24 | 4 yrs | Cattle | Indigenous |
| 25 | 25 | 3 yrs | Cattle | Indigenous |

**Table 1:** List of blood samples collected for cytokine gene expression in JD positive and healthy animals.

## Separation of PBMCs for RNA Isolation

Heparin vials were used to take blood samples. PBMC were separated as per the method of [1] using a density gradient centrifuge and Histopaque, with a few alterations. Briefly, 4 ml of blood was progressively placed over 2 ml of Histopaque -Sigma (density -1.077g/l) in sterile centrifuge tube and was centrifuged at 1600 RPM for 40 minutes. The lymphocyte layer (PBMC) (white ring at the junction) was taken using a sterile pipette and placed in a different centrifuge tube. The lymphocytes were centrifuged at 1000 rpm for 10 minutes after being washed three times with sterile PBS (pH 6). With RPMI 1640 medium containing 10% FCS, the final wash was completed. Finally, 1 ml of the same medium was used to suspend the cells. Using the trypan blue dye exclusion method, the viability of the cells was determined. In each well of a 96-well flat bottom plate, 250 µl of this cell suspension was added. 5 microliters of Johnin Purified Protein Derivative (PPD) were also added followed by incubation of 2 hours at 37 C in a humid environment with 5% $CO_2$. Centrifugation at 10,000 rpm for 10 minutes was done to remove the cells from the RPMI 1640 media after incubation. The cells were then subjected to extraction of RNA by using Relia Prep™ RNA Cell Mini prep system (Promega) kit.

## RNA extraction and determination of RNA Yield, Quality and purity.

The RNA was extracted using the ReliaPrepTM RNA Cell Miniprep system kit from Promega according to the instructions of the manufacturer. To minimize RNA degradation, all procedures were carried out on mini-cooler or ice. The yield of total RNA was calculated. Spectrophotometer was used to calculate the purity from the relative absorbance at 230, 260, and 280 nm (i.e., A260/A280 and A260/A230).

## cDNA Synthesis

cDNA from RNA was synthesized by using Promega kit Improm II Reverse transcriptase system.

## Cytokine gene expression of IFN-γ and TNF-α

TaqMan Real time PCR was performed with minor modifications for gene expression of cytokines as per [9]. Table 2 lists the primer and probe sequences for the genes encoding IFN-γ, and TNF-α. The master mix and *M. avium paratuberculosis*-specific primers and probes were purchased from Applied Biosystems (ABI) and IDT Ltd, respectively.

| Cytokine | Primer | Probe |
|---|---|---|
| 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 |

**Table 2:** List of primers and probes used in real time PCR.

## Taq Man Real-time assay protocol and analysis

Real Time -PCR was performed with Applied Biosystems (ABI) Step one Plus PCR System. Table 3 provides the methodology for the reaction mixture made for the test and table 4 lists the cycling conditions. As per step one plus RT- PCR software, analysis of the TaqMan assay was done.

## Results

Bovine PBMC's stimulated with Johnin Purified Protein Derivative (PPD) were used to study the cytokine gene expression of IFN-γ and TNF-α in JD positive animals. A total of 20 blood samples which were detected positive based on IS900 PCR were subjected to TaqMan Real Time PCR by using GAPDH as endogenous control. Blood sample from 5 healthy animals were chosen as the reference samples

| S. No. | PCR Components | Volume used per reaction |
|---|---|---|
| 1 | TaqMan Master Mix, 2X | 10 µl |
| 2 | Forward Primer | 1 µl |
| 3 | Reverse primer | 1 µl |
| 4 | Probe | 1 µl |
| 5 | Nuclease free water | 7 µl |
| 6 | DNA template | 2 µl |
|  | Total Volume | 20 µl |

**Table 3:** TaqMan real-time PCR reaction mixture for the gene expression of cytokines.

| 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 |

**Table 4:** Cycling conditions for TaqMan qRT-PCR assay.

## Gene expression of TNF-α

In the present study, enhanced gene expression of TNF alpha was seen only in five animals. Ct values ranged from 27, 28, 32, 34 and 34 in JD positive animals as compared to the Ct values 37, 36, 36, 38 and 38 in samples from healthy animals. Figure 1 provide an amplification plot of the gene expression of TNF-α and figure 2 represents the bar diagram showing the R.Q values of the samples. Five samples showed up-regulation having RQ values of (1.45, 1.71, 4.4, 6.45, 1.24) while rest of the samples showed down-regulation having RQ values (0.89, 0.06, 0.13, 0.12, 0.87, 0.07, 0.06, 0.08, 0.11, 0.04, 0.95, 0.67, 0.42, 0.12, 0.06, 0.04, 0.04, 0.01 and 0.08) when compared to the reference sample taken from the healthy animals .

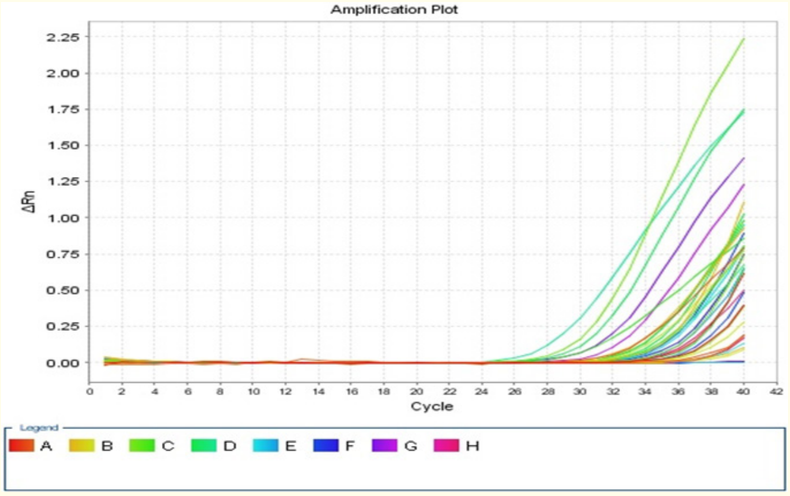

**Figure 1:** Amplification plot of cytokine gene expression of TNF-α using qPCR TaqMan Assay.

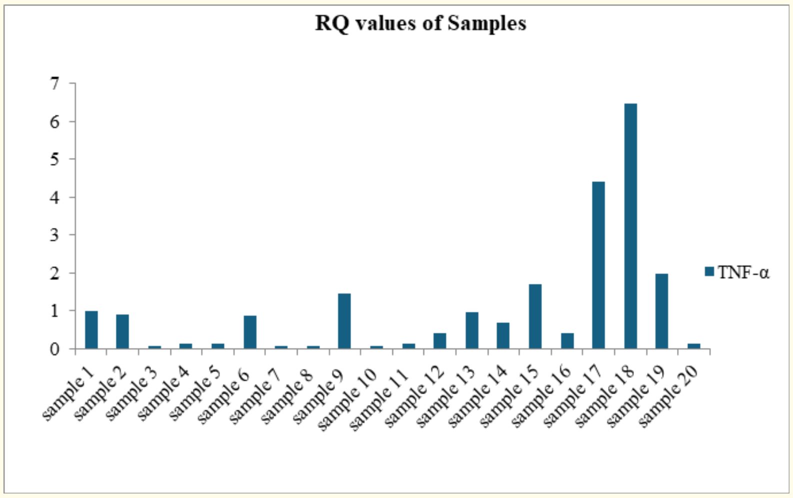

**Figure 2:** TNF-α expression profile in JD positive animals.

## Gene expression of IFN-γ

Figure 3 represents the amplification plot of IFN-γ in JD positive animals and in samples from healthy animals. Figure 4 represents the bar diagram showing the R.Q. of the samples. IFN- **γ expression was found to be up regulated in 11 animals having RQ** (5.9, 4.9, 1.6, 2.4, 4.3, 3.5, 1.5, 1.6, 4.6, 1.1 and 5.3) and down-regulated in rest of the animals with RQ (0.09, 0.11, 0.05, 0.04, 0.93, 0.33, 0.44, 0.01, 0.06, 0.01, 0.05, 0.01 and 0.01).

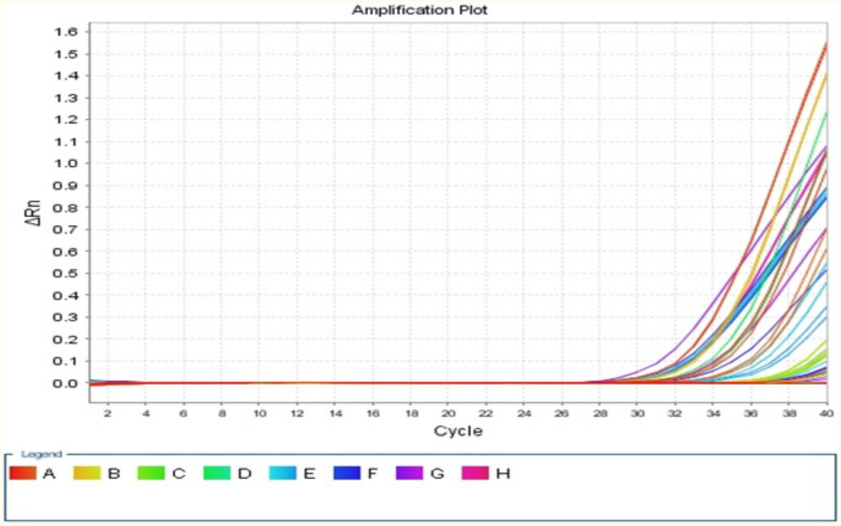

**Figure 3:** Amplification Plot of cytokine gene expression of IFN-γ using qPCR TaqMan Assay.

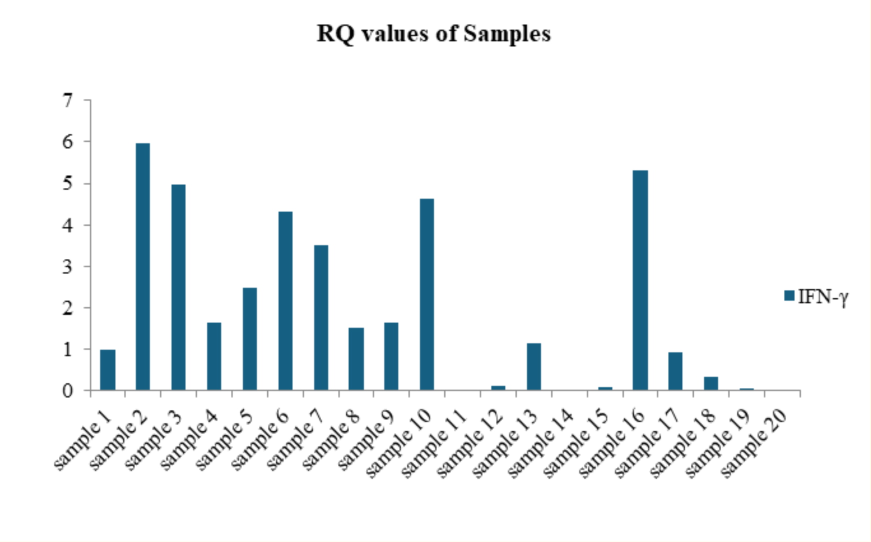

**Figure 4:** IFN-γ expression profile in JD positive animals.

## Discussion

In the present study gene expression for the cytokines TNF- α and IFN – γ from the blood samples detected positive with JD by IS900-PCR were analysed and compared to healthy animals detected negative for JD. Cellular immunity plays a key role in the host immunological response to *Mycobacterium avium* subspecies *paratuberculosis* in which cytokines produced by Th1 cells are particularly important.

The pro-inflammatory cytokines like IFN-γ and TNF-α, produced by Th1-like T cells, seem to be quite common during early infections with *Mycobacterium avium* subspecies *paratuberculosis* (MAP). These cytokines have been found to promote the disease-related granuloma formation which controls the disease progression and are in charge of the early response to MAP. One type of study conducted by Smeed., *et al.* [8] revealed that the levels of pro-inflammatory cytokines TNF-α, IL-1, IL-8 and IFN-γ were increased and stated it as evidence for ongoing inflammatory lesions during the progression of disease. Another study conducted by Li., *et al.* [6] observed the cytokine gene expression in illeal tissue of animals infected with paratuberculosis and evaluated the effects of pro-inflammatory cytokines like IL-1, IFN-γ and TNF–α. IFN-γ and IL-1 were found to be expressed more in cattle infected with the disease than the non-infected control animals. Karcher., *et al.* [4] conducted a study in which the release of IFN-γ, TNF-α, and IL-12 cytokines were studied and it showed that these cytokines were responsible for the early infection with MAP and were responsible for the development of granulomas associated with the disease.

One of the main immunological responses, characteristic of the infections caused by mycobacterium is the increased production of IFN- γ. A change from a cell-mediated (Th1) response to a non-protective antibody-mediated (Th2) response marks the progression of the disease from a subclinical or asymptomatic stage to a clinical stage of infection.

## Conclusion

To conclude in the context of the infection the pro inflammatory cytokines: TNF - α and IFN -γ produced by Th -1 cells are found to be upregulated in animals affected with Jhones disease as being the main cytokines produced during early infections caused by *Mycobacterium* which have been found to promote the disease-related granuloma formation. These JD positive animals were compared to healthy animals which were free of the disease. In the present study the gene expression of IFN- γ was found to be more upregulated than TNF– α in the diseased animals as it considered the main cytokine and characteristic of the infection involved in immune response against Mycobacteria. Apart from this it is necessary for activating macrophages which are crucial for controlling the intracellular pathogens. These cytokines are quite common during early infections with *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and have been found to promote the disease-related granuloma formation which controls the disease progression and are in charge of the early response to MAP.

## Acknowledgements

## Animal ethics statement

The present study was conducted under the approval of CPCSEA (GADVASU/CPCSEA/20/03/2019).

## Conflict of Interest

The authors report no conflict of interest.

## Bibliography

1. Böyum A., *et al*. “Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1g”. *Scandinavian Journal of Clinical and Laboratory Investigation. Supplementum* 97 (1968): 77-89.
2. Coussens PM., *et al*. “Mycobacterium paratuberculosis and the bovine immune system”. *Animal Health Research Reviews* 2.2 (2001): 141-161.
3. Ganusov VV., *et al.* “Evaluating contribution of the cellular and humoral immune responses to the control of shedding of *Mycobacterium avium* spp. *paratuberculosis* in cattle”. *Veterinary Research* 46.1 (2015) :46-62.
4. Karcher E., *et al*. “Modulation of cytokine gene expression and secretion during the periparturient period n dairy cows naturally infected with Mycobacterium avium subsp. paratuberculosis”. *Animal Industry Report, ASL R2198, AS653* (2007).
5. Khalifeh MS and Stabel JR. “Effects of gamma interferon, interleukin-10, and transforming growth factor beta on the survival of Mycobacterium avium subsp. Paratuberculosis in monocyte-derived macrophages from naturally infected cattle”. *Infection and Immunity* 72.4 (2004): 1974-1982.
6. Li L., *et al*. “The complete genome sequence of *Mycobacterium avium* subsp. *paratuberculosis”. Proceedings of the National Academy of Sciences of the United States of America* 102.35 (2005): 12344-12349.
7. Roussey JA., *et al*. “Regulatory T cells and immune profiling in Johne’s disease lesions”. *Veterinary Immunology and Immuno Pathology* 181 (2016) 39-50.
8. Smeed JA., *et al*. “Differential cytokine gene expression profiles in the three pathological forms of sheep paratuberculosis”. *BMC Veterinary Research* 3.18 (2007).
9. Widdison S., *et al.* “Cytokine expression profiles of bovine lymph nodes: Effects of Mycobacterium bovis infection and bacilli Calmette–Guerin vaccination”. *Clinical and Experimental Immunology* 144.2 (2006): 281-289.