RESEARCH ARTICLE

Cytokine Profiling Associated with Bovine Tuberculosis

Gurleen Kaur[1], Gursimran Filia[2]*, Geeta Devi Leishangthem[3], Vishal Mahajan[2]

Abstract

Cytokine expression in bovine tuberculous tissues was estimated and compared to that of non-infected healthy tissue samples using ELISA. Twenty tissue samples suspected of bovine tuberculosis were collected and subjected to a preliminary acid-fast staining technique followed by molecular detection using IS6110 PCR. The expression of anti-inflammatory cytokines interleukin IL-4 and IL-13, immunomodulator IL-10, and the pro-inflammatory cytokines tumour necrosis factor (TNF-α) and interferon-gamma (IFN-γ) were estimated in the tissue samples positive for bovine tuberculosis. The dominance of pro-inflammatory cytokines (IFN-γ and TNF-α) over the anti-inflammatory cytokines (IL-4 and IL-13) in the tuberculosis-infected tissues indicated that the pro-inflammatory cytokines played a crucial role in the tissue damage occurring in the disease. Immunomodulatory cytokine IL-10 levels were also significantly increased indicating its role in the maintenance of the balance of the cytokines. Thus, the present study revealed that cytokines play a major role in the immunopathogenesis of bTB.

Key words: Bovine tuberculosis, Cytokines, Enzyme-linked immunosorbent assay, Polymerase chain reaction,

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Introduction

Immunity to mycobacteria is dependent mainly on a cell-mediated response involving macrophages, dendritic cells, and an adaptive T-cell response. The functions of these cells are modulated by low molecular weight regulatory proteins called cytokines and other mediators (Flynn and Chan, 2001). Cytokines are secreted by lymphocytes, effector cells, and antigen-presenting cells and act as intercellular communication signals in a variety of processes including tissue repair, stress, haematopoiesis, inflammation, immunity, and embryonic development.

Cytokines play a wide variety of roles in bovine tuberculosis. Pro-inflammatory cytokines mediate the T-helper type-1 (TH1) responses involving macrophages, dendritic cells, and T-cells. Interferon-gamma (IFN-γ) plays a critical role in the control of mycobacterial infection, while other pro-inflammatory cytokines, such as IL-12 and tumor necrosis factor-alpha (TNF-α) play a role in the TH1 response (Flynn and Chan, 2001). During *M. bovis* infection, T-Helper type-2 (TH2) responses characterized by the production of anti-inflammatory cytokines such as IL-4, IL-5, and IL-13 are thought to inhibit type-1 T cell (TH1) responses (*i.e.,* TNF-α, IL-6 response) and also contribute to the granuloma formation. An understanding of the granuloma-level cytokine response is necessary to understand tuberculosis pathogenesis (Palmer *et al*., 2016). Thus, the present study was conducted to analyze the cytokine expression in bovine tuberculosis-affected lungs/lymph nodes.

[1]Department of Veterinary Microbiology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab-141001, India

[2]Animal Disease Research Centre, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab-141001, India

[3]Department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab-141001, India

Corresponding Author: Dr. Gursimran Filia, Animal Disease Research Centre, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab-141001, India
e-mail: harpalfilia@rediffmail.com

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Materials and Methods

Collection of Samples

Lungs (20) were collected from dead animals showing tuberculous lesions and five tissue samples from apparently healthy animals which were submitted for post-mortem examination at Department of Veterinary Pathology, GADVASU, Ludhiana (India), and also from field outbreaks. The tissue samples were collected in sterile containers and were stored at -20°C till molecular studies were carried out. Collection of the samples was done with utmost care following the safety measures. Proper face mask, gloves, apron and shoe covers were worn while collecting the tissue samples to avoid any contact with the affected tissue.

Preparation of Impression Smear

Tissue impression smears were made immediately after the collection of samples and were subjected to Ziehl-Neelsen staining for demonstration of acid-fast bacilli (Filia *et al*., 2016).

Polymerase Chain Reaction

DNA was extracted from each lung tissue sample using the QIAmp Blood and tissue DNeasy kit (Qiagen). All the DNA samples were stored at -20˚C till further use. Identification of *Mycobacterium tuberculosis* complex (MTC) was done using a specific pair of primers, INS1/INS2, which amplify an insertion sequence *IS6110* for 245 bp fragment in MTC. PCR was performed as per Figueredo *et al.* (2009) with some modifications using the already designed INS1 (5'CGTGAGGGCATCGAGGTGGC3') and INS2 (5'GCGTAGGCGTCGGTGACAAA 3') primers. Briefly, a reaction volume of 25 µL was made containing 13 µL of Taq PCR Master Mix (Qiagen), 1 µL of each forward & reverse primer (10 pmol/µL), 5 µL nuclease-free water and 5 µL of DNA template of the sample. Positive control was obtained from Animal Disease Research Centre, GADVASU, Ludhiana. For negative control, 5 µL of NFW was used as the template. Thermal cycling was performed in T. Gradient Thermocycler (Biometra, Germany) and cycling conditions followed were: initial denaturation at 94˚C for 5 min, followed by 35 cycles each of denaturation at 94˚C for 1 min, annealing at 62˚C for 1 min, extension at 72˚C for 1 min, and final extension at 72˚C for 7 min. For analysis of the PCR products, gel electrophoresis was performed in 1.5% agarose gel and visualized in the Gel Documentation system (BioRad). Duration for UV exposure was optimized and photographs were taken. Amplicons of 245 bp were considered positive for MTC.

Cytokine Expression in Bovine Tuberculosis Affected Lungs and Lymph Nodes

Preparation of samples & Protein estimation

The tissue samples (10 mg) were homogenized in 1 µL ice-cold phosphate buffer saline (pH 7.4) using tissue homogenizer with a Teflon pestle at 4°C. The resultant homogenate was used to estimate proteins and cytokines by ELISA.

Protein estimation was done in each tissue homogenate using Thermo Scientific™ Pierce™ BCA Protein Assay Kit as per manufacturer's protocol. Each standard or unknown sample (25 µL) was pipette into a microplate well. A working reagent (200 µL) was added to each well and plate mixed thoroughly on a plate shaker for 30 seconds. The plate was then covered and incubated at 37°C for 30 min (Sharma *et al.,* 2020). Later, the plate was cooled to room temperature. Absorbance of a purple-coloured reaction mixture formed was measured at 562 nm on a plate reader.

ELISA

The cytokine IFN-γ was estimated using Thermo scientific bovine INF-γ ELISA kit and the cytokines TNF-α, IL-10, IL-4, and IL-13 were estimated using a commercially available kit (Biocodon ELISA Kit) as per the manufacturer's protocol. The absorbance (OD) was measured at 450 nm wavelength within 10 min after addition of stop solution in an ELISA Reader (Thermo Scientific; Multiskan).

S No.	Name of the kit	Company
1	Pierce™ BCA Protein Assay Kit	Thermo Scientific™
2	Thermo scientific bovine INF-γ ELISA kit	Thermo Scientific™
3	Bovine Interleukin 10, IL-10 ELISA Kit	Biocodon USA
4	Bovine Interleukin 13 (IL-13) ELISA Kit	Biocodon USA
5	Bovine Interleukin 4, IL-4 ELISA Kit	Biocodon USA
6	Bovine Tumor necrosis factor α, TNF-α ELISA Kit	Biocodon USA

Statistical Analysis

For cytokine estimation, data were expressed as Mean ± SD and student t-test was employed to calculate significance at 5% level (Gupta *et al.,* 2016).

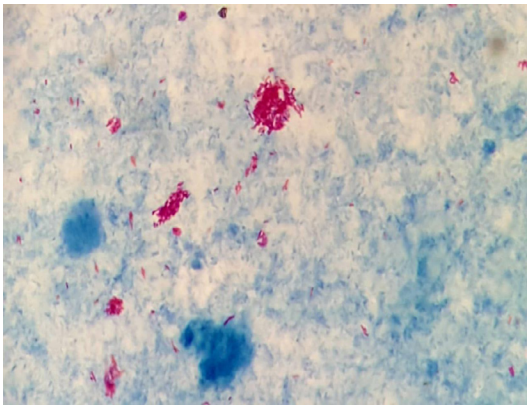

Fig. 1: Acid-fast stained bacilli with ZN staining

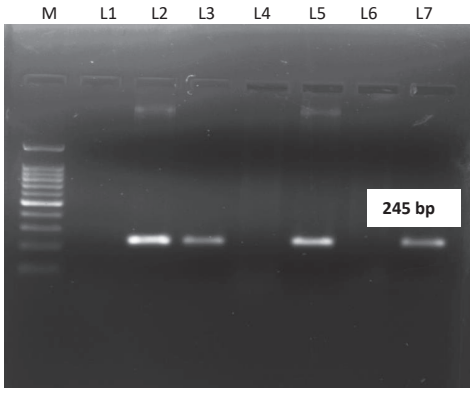

Fig. 2: Agarose gel electrophoresis of the amplified IS6110 gene of MTC at 245 bp using INS1/INS2 primers. M: DNA 100bp ladder, L1: Negative control, L2: Positive control, L3-L7: samples

Results and Discussion

Detection of Acid-Fast Bacilli

Out of 20 suspected samples, 13 impression smears were positive for acid-fast bacilli using Zeihl-Neelsen (ZN) staining. Acid-fast bacilli were found to be located mainly within the caseous necrotic centre of granulomas and also within macrophages and multinucleated giant cell (Fig. 1).

In the smears positive for acid-fast bacilli, these slow-growing mycobacteria got adapted within the macrophages for survival and this capacity of the organism to persist against the potent cellular response was the reason for the chronic inflammatory infection in the host (Saunders and Britton, 2007). According to Filia *et al.* (2016), the fastest and simplest method of confirming mycobacterial infection was staining the smears, prepared from the suspected samples, with the ZN stain to demonstrate the acid-fast tubercle bacilli under the microscope. ZN staining could be helpful in preliminary confirmation of the disease after the regular post-mortem inspection.

IS6110 Polymerase Chain Reaction

A total of 11 samples were detected positive by PCR after amplification of the DNA using primers specific for *IS6110* sequence targeting 245 bps fragments for MTC members (Fig. 2). PCR technique offered high sensitivity and has been successfully used for diagnosing bTB in several types of naturally infected organic material such as tissue, blood, and nasal exudates (Coetsier *et al*., 2000; Gómez-Laguna *et al*., 2010). Filia *et al.* (2016) detected 19 (15.70%) animals out of a total of 121 animals to be positive for MTC by PCR on blood samples of cattle.

The use of PCR assay to detect *M. bovis* in tissue homogenates provides a more rapid method for confirmative diagnostic test results to field veterinarians than culture (Thacker *et al*., 2011). However, one of the problems with detecting MTC directly from lesions that are compatible with tuberculosis was that tissues generally exhibited strong fibrosis and calcification, which decreased the access to the mycobacterial DNA (Liebana *et al*., 1995). PCR technique offered high sensitivity and has been successfully used for diagnosing bTB in several types of naturally infected organic material such as tissue, blood, and nasal exudates.

Cytokine Expression

In the present study, there was a significant increase in the level of pro-inflammatory cytokines TNF-α and INF-γ, whereas a mild increase was observed in the level of anti-inflammatory cytokines IL-4 and IL-13. A significant increase in the level of immune modulatory cytokine IL-10 was observed with respect to control. However, both pro-inflammatory and anti-inflammatory cytokines along with IL-10 (immune-modulator) increased in TB-positive animals as compared to that of healthy animals.

A significant rise in the levels of IFN-γ ($p< 0.05$) was found in the TB-infected animals (1441.69±607.12 pg/mg protein), as compared to the levels in normal animals (665.08±128.62 pg/mg protein). This result was in accordance to a study conducted by Rhodes *et al*. (2000), who reported an increased IFN-γ response in all the experimentally infected animals by four weeks post-infection and these responses continued to increase up to 6 to 8 weeks post-infection. An increase IFN-γ response was observed in 15 out 21 naturally infected cattle.

TNF-α is a key determinant of granuloma formation and integrity. In the present study, an increased level of TNF-α ($p<0.05$) was observed in TB-infected tissues (396.37±96.40 pg/mg protein) as compared to the levels in the healthy tissue levels (255.36±50.319 pg/mg protein), which indicated the role of TNF-α in granuloma formation in the affected lung tissues. Palmer *et al*. (2016) reported a greater expression of TNF-α in trachea-bronchial lymph node granulomas compared to uninfected lymph nodes.

The expression of IL-10 was significantly higher in the diseased tissues (585.17±147.39 pg/mg protein) as compared to healthy animals (359.78±43.50 pg/mg protein). A mild increase in the levels of IL-4 and IL-13 cytokines ($p<0.05$) was observed in the tissues infected with mycobacterial organisms as compared to the healthy animal tissues. The statistical analysis of cytokine expression is given in Table 1.

A number of studies have been conducted to understand host immune response in peripheral blood cells, but studies to investigate cytokine response of lungs and lymph nodes at the site of mycobacterial infection in cattle and buffalo are very scarce. The present study was performed to analyze the levels of cytokines at the site of mycobacterial infection in the affected lung samples. The results of the study were in accordance to Witchell *et al*. (2010), who diagnosed that *M. bovis* infected lymph nodes had significantly higher expression of INF-γ and TNF-α, and also increased level of IL-10 was seen which was considered as an immunological marker for disease progression. The authors further observed that there was a large fold increase in IFN-γ in response to *M. bovis* infection and was significantly higher than the increase in TNF-α and IL-10. On the contrary, Widdison *et al.*

(2006) observed suppression of IL-4, IL-10, TNF-α response when compared to normal healthy samples and no change was seen in INF-γ, which was contrary to the results of the present study.

IFN-γ is a dominant pro-inflammatory cytokine during tuberculosis infection. One of the major roles of IFN-γ is to activate macrophages which then produce an array of pro-inflammatory cytokines, including both IL-1β and TNF-α that were thought to play an important part in the control of mycobacterial infection (Kindler *et al*., 1989). Gupta *et al*., (2016) conducted a study on cytokine expression in natural cases of 32 bovine lymphadenopathies. In the case of bovine tuberculosis, there was a significant increase in the levels of INF-γ, parallel to the results obtained in the present study, indicating a paramount role of IFN-γ in mycobacterial immunity. Higher IFN-γ expression suggested that localized T-cells were primed by macrophages, which in turn produced cytokine leading to the production of activated macrophages having mycobactericidal activity.

TNF-α is another pro-inflammatory cytokine produced primarily by macrophages, but also produced by lymphocytes, mast cells, and fibroblasts. It promotes anti-mycobacterial activity by enhancing phagosome-lysosome maturation. It also helps in the presentation of antigens to the T-cells, enhancing CD4+ T cell response (Ramachandra *et al*., 2009). TNF-α plays a key role in the initiation of the inflammatory response, its regulation and progression and is also required for the induction of apoptosis of infected macrophages in response to mycobacterial infection (Balcewicz-Sablinska *et al*., 1998).

IL-10 is produced by haematopoietic and non-haematopoietic cells in response to MTC infection (Abdalla *et al*., 2016). In the present study the levels of IL-10 were observed to be significantly increasing ($p<0.05$) indicating an important role of IL-10 in maintaining immunological balance for controlled bacterial burden and disease pathology in MTC infections. This increased expression of IL-10 is associated with the decreased ability of macrophages to restrict the growth of intracellular Mycobacterium (Jamil *et al*., 2007; Bilenki *et al*., 2010). IL-10 can also inhibit the IFN-γ mediated macrophage activation. Wang *et al*. (2011) also showed that IL-10 expression is increased in tuberculosis-infected cattle (8.74-fold) as compared to the healthy group (2.90-fold) of animals. IL 10 levels increased in MTC infected animals in the present study. IL-10 plays a fundamental role in maintaining host homeostasis, ensuring the fine equilibrium between pro- and anti-inflammatory immune response required to achieve an effective clearance of infecting pathogens and preventing, at the same time, tissue damage occurrence (Ouyang and O'Garra 2019). In physiological conditions, IL-10 production is under a highly dynamic and finely balanced modulation to orchestrate the different immunological activities in a cell-specific manner and to control the inflammatory response force and duration (Carlini *et al*., 2023).

IL-4 is a classic anti-inflammatory TH2 cytokine that down-regulates TH1-mediated responses (*Lazarski et al*., 2013). Although it is known that IL-4 inhibits TH1 functions, but it might be involved in the killing of *Mycobacterium* by down-regulating neutrophil superoxide production (Abramson and Gallin, 1990). Thus, IL-4 might prevent the development of mycobacterial infection and play a significant role in defence against mycobacteria, although INF-γ and TNF-α play major roles in it (Sugawara *et al*. 2000). Another implication could be that IL-4 led to induction of cytotoxic T cells. Increased expression of this cytokine results in the accumulation of monocytes and eosinophils in the lung granuloma.

IL-4 and IL-13 could undermine TH1-mediated immunity and drive inappropriate alternative activation of macrophages (Rook, 2007). IL-13 may play a modulatory role in autophagy, which is an important homeostatic mechanism for intracellular degradation and has a protective function during mycobacterial infection (Harris *et al*., 2007). Increased levels of IL-13 from whole blood mRNA was observed in patients who were latently infected with *M. tuberculosis* as compared to the uninfected controls (Dhanasekaran *et al*., 2013). Similar findings were observed in the present study with a mild increase in the levels of IL-13 in the infected tissues as compared to the control uninfected tissues.

Conclusion

In the present study, there was a significant increase in the level of pro-inflammatory cytokines TNF-α and INF-γ, whereas a mild increase was observed in the level of anti-inflammatory cytokines IL-4 and IL-13 in TB-positive animals. A significant increase in the level of immune modulatory cytokine IL-10 was observed with respect to control. However, both pro-inflammatory and anti-inflammatory cytokines along with IL-10 (immune-modulator) increased in TB-positive animals as compared to that of healthy animals. The study indicated a stronger pro-inflammatory immune response in infected lung tissues. The dominance of pro-inflammatory cytokines (IFN-γ and TNF-α) over the anti-inflammatory cytokines (IL-4 and IL-13) in the tuberculosis-infected tissues indicated that the pro-inflammatory cytokine played a crucial role in the tissue damage occurring in the disease. Immunomodulatory cytokine IL-10 levels were also significantly increased indicating its role in the maintenance of the balance of the cytokines. Thus, the present study revealed that cytokines play a major role in immunopathogenesis of bTB.

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