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Analysis of Toll like Receptors and Interleukins Expression Profile in *Mycobacterium avium* sub sp. *paratuberculosis* Infected Cattle

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Dissecting variation in gene expression between infected and healthy controls for the genes involved in pathways of immune response to Mycobacterium avium subsp. Paratuberculosis (MAP) infection will allow us to test the hypothesis that variation in expression of these genes influences the disease status in animals. Thus the objective of this study was to analyze differential expression profiles of TLR2, TLR4, IFN-ã, IL2, IL8, IL16 and TNF genes using qRT-PCR in Mycobacterium avium subsp. Paratuberculosis infected cattle vis a vis healthy controls and to assess whether any inherent gene expression patterns are visible for these key innate immune response genes. In the present investigation, distinctive gene expression patterns between PBMCs of healthy cattle and those exposed to MAP. Highest fold change expression was found in IFN-ã gene which was significantly upregulated (7.24 fold, p = 0.0367) in PBMC's of MAP infected cattle vis a vis healthy controls Expression of TLR2 and IL8 genes were also significantly upregulated in PBMC's of MAP infected cattle as compared to healthy cattle. Whereas TNF and IL2 genes revealed significant repression in mRNA expression in PBMC's of MAP infected cattle relative to healthy controls. However in case of TLR4 and IL16 genes, non significant differences were observed for mRNA expression between PBMC's of MAP infected cattle and Healthy controls. Our results indicate that differences at mRNA level can be utilized in biomarker based segregation of infected and healthy and understanding host-pathogen interactions.

Key words: Expression, MAP, Genes, Immune response, PBMC.

Mycobacterium avium sub species *paratuberculosis* (MAP) is a facultative intracellular pathogen that causes Johne's disease (JD), a chronic granulomatous inflammation of the intestine characterized by persistent diarrhea, progressive wasting, and finally death[1]. Johne's disease has a global presence and is primarily a disease of domesticated ruminants, including cattle, sheep and goats[1,2]. The host range for Johne's disease has been reported to include wild ruminant species, such as deer as well as non-ruminants, such as wild rabbits and their predators[3,4]. JD has major negative effects on livestock production, both in terms of economics and on animal welfare. Loss due to reduced milk yields alone in case of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infected cows were reported to be Rs 54,442.5 /cow/lactation in India[5]. It was estimated that MAP costs the US dairy industry $200 to $250 million annually due to increased cow replacement costs and reduction in milk production[6] and also decreased fertility in

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high-shedding animals[7]. Furthermore, it has been suggested that MAP can exist within the tissues of animals for years without causing clinical disease[8]. Subclinically or clinically infected animals shed MAP in faeces and milk, enabling dissemination to susceptible calves, the environment, and in milk. Role of MAP in public health as the causative agent of Crohn's disease has also long been discussed. Due to the histopathological features of Crohn's disease closely resembling those found in animals with the paucibacillary form of Johne's disease, it has been suggested that the two diseases shared the same aetiology[9,10]. MAP is mostly acquired orally in cattle and other ruminants and often in young calves[11] although intra-uterine transmission of MAP occurs[12,13]. Post initial infection, most animals begin to develop a T-cell response, characterized by release of the proinflammatory cytokines gamma interferon (IFN- γ) and tumor necrosis factor alpha (TNF-α), as well as production of interleukin-2[14]. These cytokines in turn lead to recruitment and activation of cytotoxic $CD8^+$T cells and other immune components, including neutrophils and additional activated macrophages, to sites of *M. paratuberculosis* infection[15,16]. It is followed by recruitment of cytolytic $CD8^+$T cells to the site of infection comprise development of an acquired T-helper cell type 1 (Th1) like immune response against *M. paratuberculosis*. Rapid development and continued maintenance of this response is a critical factor in controlling mycobacterial infections in a variety of species[15,17]. Control of the disease in the early stages is associated with Th1 response via the release of Th1-type inflammatory cytokines, interleukin-2 (IL-2), TNF, IFN- γ, and IFN-β, which direct cell-mediated immunity[18]. Among them, IFN-γ plays a pivotal role in activation of macrophages and their subsequent ability to kill MAP[19]. Infected cattle initially develop an early and appropriate pro-inflammatory Th1 and cytotoxic immune response to MAP antigens. However, this response typically declines during the long subclinical phase of infection, and a Th2 response becomes predominant, but is ineffective in controlling the bacteria[20]. MAP is able to arrest normal phagosome maturation in infected cells, being able to persist and multiply within the infected host phagosome.

Classical control strategies based on management restrictions to reduce transmission, culling of infected animals and vaccination has not been able to eradicate Johne's disease from infected herds. Selective breeding for less susceptibility to disease may be a useful additional tool to contribute to control of the disease. Heritability of susceptibility to Johne's disease in cattle has been shown to vary from 0.041 to 0.159[21,22,23] demonstrating genetic variation for susceptibility to MAP in dairy cattle but the understanding of genes contributing to the genetic variance is far from complete. SNP associated with MAP-specific antibody response in milk were also identified distributed over regions of chromosome 4, 15, 18, and 28[24]. Genes, coding for proteins with very specific and unique roles in immune responses are potential strong candidates for investigating genetic basis of disease resistance. Gene expression levels of IFN-γ, IL2, NFKB1, TLR 2, TNF have been reported to be significantly different in naturally MAP infected as compared to healthy animals[25,26]. Further it has been reported that *invivo / in vitro* infection with MAP leads to differential expression of IFN-γ, IL2, NFKB1, TLR 2, TNF, IL6, IL8, IL10 and associated interleukins[27,28,29]. Gene-expression profiling studies suggested the hypothesis that inherent gene-expression profiles in peripheral blood mononuclear cells (PBMCs) from *M. paratuberculosis*-infected cattle may be different than expression profiles in PBMCs from uninfected controls. Thus it may be possible to identify an *M. paratuberculosis* infection signature through transcriptional profiling of peripheral immune cells. Also delineation of groups of genes showing inherently different expression in PBMCs from *M. paratuberculosis*-infected cattle relative to PBMCs from uninfected controls might highlight important interactions between this pathogen and the host immune system. Therefore the objective of this study was to analyze differential expression profiles of TLR2, TLR4, IFN-γ, IL2, IL8, IL16 and TNF genes in *Mycobacterium avium* subsp. *Paratuberculosis* infected cattle vis a vis healthy controls.

MATERIALS AND METHODS

Experimental Animals

The infected and control cattle used in this study were all multiparous non-descript cows

ranging in age from 24 to 60 months and were all housed in Sri Mataji Gaushala, Barsana, Mathura. Johnin skin test, ELISA (PARACHEK® kit) and Faecal ZN staining were used for diagnosis of MAP infected animals. In Skin test for Delayed type hypersensitivity (DTH), intradermal inoculation of 0.1 ml of Johnin PPD antigen on neck region is carried out. The skin thickness was measured with calipers before and 72 hours after inoculation. Increase in skin thickness of over 4 mm was regarded as indicating the presence of DTH[30]. Ziehl–Neelsen-stained smears of faeces were examined microscopically for presence of MAP. A presumptive diagnosis of *paratuberculosis* was made if clumps (three or more organisms) of small (0.5–1.5 µm), strongly acid-fast bacilli were found. A commercial solid-phase indirect enzyme immunoassay kit, PARACHEK (Prionics) was used to detect serum antibodies developed to MAP antigens in active stage of infection. Putative cross-reacting antibodies in uninfected animals to other mycobacteria were removed priori by absorption of serum with M. phlei. Six animals which tested positive for all these tests and six cattle which tested negative for all these tests were included in further investigation.

Sample collection and Peripheral blood mononuclear cells (PBMC) isolation

5 ml of Blood was collected from six positive and six negative cattle in sterile tubes containing heparin as anticoagulant. PBMC were isolated from fresh heparinized blood by density gradient centrifugation method following standard protocols using Histopaque (Sigma, USA) with specific gravity 1.077 g/ml. Briefly, blood was slowly layered over equal volume of Histopaque and centrifuged at 400g for 45 min and resulting interface containing PBMCs was collected and washed twice in sterile Phosphate Buffered Saline (PBS), and finally resuspended in RPMI 1640 containing 10% heat-inactivated fetal bovine serum. Cell viability was determined by Trypan Blue staining.

Extraction of RNA and cDNA conversion

RNA was extracted from MAP positive and negative PBMCs by using Trizol reagent (Invitrogen, USA.) as per manufacturer instructions. All RNA samples were treated with RNase-free DNase I (Thermo Scientific, USA). The quantity and quality of extracted total RNA were estimated by UV spectrophotometry and electrophoresis on 1.2% native agarose gels. To evaluate the gene expression profiles of PBMCs, total RNA (2 µg) from PBMCs of each animal were used as templates in reverse transcription reactions, using a commercial kit, High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and oligo(dT) as primer. Control reactions, lacking reverse transcriptase, were performed for every RNA sample. cDNA samples were stored at -20^{0}C until use.

Quantitative reverse transcription-PCR (qRT-PCR) analysis

Real-time qRT-PCRs were performed by using an CFX Real-Time PCR Detection Systems (BioRad, USA), 20 ng of template cDNA, and gene-specific primers. Seven gene specific primers (TLR2, TLR4, IL2, IL8, IL16, TNF and IFNG) were used for real time expression profiling (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene to assess differential gene expression between infected and healthy controls. Maxima SYBR Green qPCR Master Mix (2X) was used for PCR amplification in a final volume of 25 µl. Real time PCR reactions were accomplished using 2 ¼l of cDNA, 300 nM each of gene specific primers and 10 ¼l Maxima SYBR Green qPCR Master Mix (2X) (Promega) with SYBR green assay. The mix was optimized for efficient and reproducible PCR. Cycling parameters were 95^{o}C for 5 min, followed by 40 cycles of 95^{o}C for 30 s and 58^{o}C for 30 s and 72^{o}C for 30 s. Fluorescence was measured at the end of the annealing/extension step. Reactions were run in triplicate for each gene and the specificity of the PCR products was verified by gel electrophoresis and melting curve analysis. Results were normalized to the gene GAPDH as endogenous control. A negative control without template (cDNA) was always kept in order to check any PCR carryover. Random samples were analyzed in the absence of reverse transcriptase so as ensure that genomic DNA contamination was not contributing to the specific cDNA amplification. Further, non-template control samples were included in each run. The qRT-PCR results for GAPDH were used to calculate differences in the template RNA levels and thereby standardize the results for the genes of interest. GAPDH was

previously selected from microarray and qRT-PCR analyses as a constitutively and moderately expressed gene in PBMCs of cattle[31]. Relative expression of each sample was calculated using the 2^{-CT} method[32]. Normalization was done for expression of target genes to the expression of reference gene. Briefly, mean Ct values were derived from replicate assays for each gene and sample. Next, for each gene and sample the mean Ct for the GAPDH reference gene was subtracted from the mean Ct for each gene assayed, giving a $^{-}$Ct value for that gene/sample. The $^{-}$Ct values for the control group were then subtracted from the respective MAP positive PBMC $^{-}$Ct values for each gene, giving a $^{--}$Ct value that represented the fold-change between MAP positive and healthy control animals. Finally, the actual fold-change values between MAP positive and healthy control for each gene were calculated as $2^{"--Ct}$. Three technical replicates were averaged and the results were reported as Mean ± SE. Results were analysed and shown as Fold change (2^{---CT}).

RESULTS

PCR amplification of selected immune response genes in PBMCs

Genes selected for the study (TLR2, TLR4, IL2, IL8, IL16, TNF and IFNG) were amplified from cDNA of PBMCs (Fig. 1) to check the specificity of the primers.

Differential expression of TLR2, TLR4, IL2, IL8, IL16, TNF and IFNG genes in Cattle infected with *Mycobacterium avium* subsp. *Paratuberculosis* vis a vis healthy controls

Five innate immune response genes under present investigation displayed differential expression between PBMC's of MAP infected cattle and healthy controls (Fig.2). Level of fold change differences in gene studied varied from -1.38 to 7.24 fold in PBMC of MAP infected cattle relative to healthy controls (Table 2). Highest fold change expression was found in IFN-γ gene. Expression of TLR2 gene was significantly upregulated (4.92 fold, p=0.0227) in PBMC's of MAP infected cattle as compared to healthy cattle. Whereas in case of TLR4 gene, non significant differences were observed for mRNA expression between PBMC's of MAP infected cattle and Healthy controls.

Expression of TNF gene was significantly downregulated (-1.90 fold, p=0.0001) in PBMC's of MAP infected cattle as compared to healthy controls. PBMC's of MAP infected cattle relative to Healthy controls. IFN-γ gene mRNA expression was found to be significantly upregulated (7.24

Table 1. Quantitative Real Time PCR primers for the selected gene fragments of Cattle

Gene Name	Chromosome No.	Sequence (5' -3')	Amplicon size (bp)
TLR2	17	F: CCATTGACAAGAAGGCCAT R: AACCCTTCCTGCTGAGTCTCAT	107
TLR4	8	F: CGAGAGCACCTATGATGCCTTT R: ATGGCCACCCCAGGAATAAA	144
IL2	17	F: CTTGCACTCGTTGCAAACG R: CAAGCTCTCCAGGATGCATACA	183
IL8	6	F: AGGTGGTGTTTGAAGCCCAT R: CACAACCTTCTGCACCCACTT	123
IL16	21	F: CGCGGTTTGAAGAATGGAAC R: TCACAGGTCCATCAGGCAAC	51
TNF	23	F: TCTACCAGGGAGGAGTCTTCCA R: GTCCGGCAGGTTGATCTCA	68
IFN-ã	5	F: TGATGGCATGTCAGACAGCA R: GGCACAAGTCATATAGCCTGACAC	51
GAPDH	5	F: CTCCCAACGTGTCTGTTGTG R: TGAGCTTGACAAAGTGGTCG	222

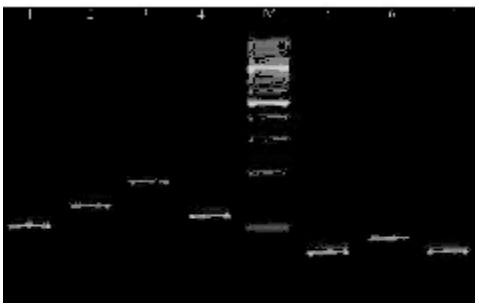
fold, p = 0.0367) in PBMC's of MAP infected cattle vis a vis healthy controls. IL2 gene revealed significant repression in mRNA expression (-1.38 fold, p= 0.0001) in PBMC's of MAP infected cattle relative to healthy controls. Expression of IL8 gene was significantly upregulated (3.48 fold, p=0.0019) in PBMC's of MAP infected cattle as compared to healthy controls. However for IL16 gene, non significant differences in gene expression were observed between PBMC's of MAP infected cattle and healthy controls

DISCUSSION

M. avium subsp. *paratuberculosis* is an intracellular pathogen that infects, and persists within, host tissues. In the contest for survival between the host and pathogen, complex cell-mediated immune responses are elicited. Dissecting variation in gene expression between infected and healthy controls for the genes involved in pathways of immune response to MAP infection will allow us to test the hypothesis that variation in expression of these genes influences the disease status in animals. Hence we focused on genes which have been previously reported to be associated with MAP infection in cattle. In the present study, an effort has been made to analyze the differential expression for the seven genes involved in immune response between MAP infected and uninfected healthy control cattle using Quantitative Real Time PCR (qRT-PCR). qRT-PCR is a highly sensitive method that allows quantification of rare transcripts and small changes in gene expression from a limited amount of sample[33]. A reference or internal control gene that is not affected by the experimental treatment is used to correct for variations due to differences in RNA quantity, as well as efficiency of reverse transcription and cDNA amplification[34]. Selected

Table 2. Significant Fold Change differences in gene expression in PBMC of MAP infected cattle relative to healthy controls

Gene	Relative Expression in MAP infected vs. Healthy cattle	P-value
TLR2	4.92 ± 0.49	0.0227
TNF	-1.90 ± 0.02	0.0001
IFN-γ	7.24 ± 0.71	0.0367
IL2	-1.38 ± 0.01	0.0001
IL8	3.48 ± 0.38	0.0019



Lane 1: TLR2 (107bp); Lane 2: TLR4 (144 bp); Lane 3; IL2 (183bp); Lane 4: IL8 (123 bp); Lane 5: IL 16 (51bp); Lane 6: TNF (68 bp); Lane: IFN-γ (51bp); M: 100 bp ladder

Fig.1. PCR amplification of TLRs and interleukins in PBMCs

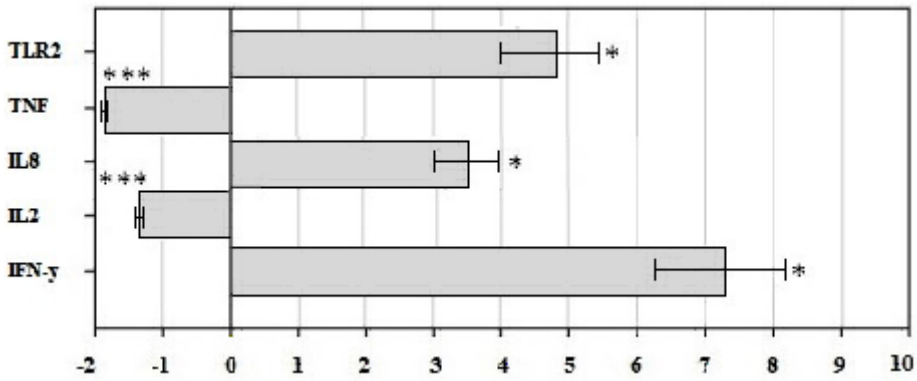


Fig. 2. Differential gene expression in PBMC of cattle infected with *Mycobacterium avium* sub sp. *paratuberculosis* relative to PBMC of healthy cattle. Error bars show standard error of mean of each gene. Statistical significance of each gene is as follow ***P≤ 0.001; *P≤ 0.05.

genes under study were based on relevant literature in cattle and other ruminants and human models of MAP, including those encoding molecules involved in pathogen recognition (TLR2, TLR4) and cytokine production (IFN-γ, TNF, IL2, IL8 and IL16). With the panel of 6 cattle per study group, the statistical significance of the mean altered gene expression within each group of cattle could be assessed with greater accuracy. Here, we demonstrate the existence of distinctive gene expression patterns between PBMCs of healthy cattle and those exposed to MAP.

TNF is a vital proinflammatory cytokine that play an important role in the innate immune response against invading M. bovis[35]. In the present investigation, qRT-PCR analysis revealed significant down regulation of TNF-α in PBMC of MAP infected cattle as compared to uninfected healthy control cattle. In agreement with our findings, expression of TNF-γ was found to be significantly lower in MAP infected than in uninfected cattle[36]. Similarly, downregulation of the proinflammatory cytokines TNFα was observed in ovine Monocyte Derived Macrophages (MDM) infected with MAP isolates[37]. The reduced response may weaken protective immunity and perpetuate infection. The reduced TNF-γ responses observed may have a negative effect on granuloma formation and function. TNF-γ plays an important role in the host defense against mycobacterium infections, including the initiation and formation of granulomas and activation of antimycobacterial killing mechanisms[38,39]. In this regard, it has been suggested that IL-10 secreted by *M. avium* subsp. *paratuberculosis*-infected macrophages suppresses TNF-α expression[40]. However, TNF gene was reported to be significant upregulated across all three studied time points post *in vitro* infection with MAP in the Monocyte Derived Macrophages (MDM) in cattle[28]. Increased levels of the cytokine TNFα mRNA was also reported in aucibacillary and multibacillary forms of Sheep *paratuberculosis*[41]. IFN-γ is reported to activate microbicidal activity in infected macrophages and also promotes the sequestration of the pathogen in granulomas-organised complexes of immune cells consisting of lymphocytes, non-infected macrophages and neutrophils that contain mycobacterial-infected macrophages and prevent the spread of bacilli to other tissues[14]. In our study, IGN-α gene was found to be significantly upregulated in PBMC's of MAP infected cattle. This finding supports the conclusion of previous reports which demonstrate that PBMCs from MAP infected cows produced significantly higher IFN-γ mRNA than produced by PBMCs from the control group[42]. Expression levels of IFN-γ were also significantly ($P< 0.1$) greater in PBMCs *invitro* stimulated with MAP than in the nil control stimulated PBMCs in clinical infected cows[25]. Higher IFN-γ mRNA expression was also observed in cows in the Tuberculoid-type lesions which occur in the early stages of *paratuberculosis*[26]. Expression of IFN-[3] was higher in section of ileal tissues taken from MAP infected as compared to ileal tissues from healthy animals[43]. Cytokines IFN-γ and TNF-α appear to modulate killing of the MAP organisms. Pretreatment of bovine monocytes with IFN-γ or high doses of TNF-α led to restriction of MAP growth in vitro[44,45]. TLRs play an important role in the recognition of components of pathogens and subsequent activation of the innate immune response, which then leads to development of adaptive immune responses. TLR2 and TLR4 could recognize BTB products and rapidly generate a defensive response involving numerous proinflammatory cytokines and Th1 cytokines to restrict the growth of intracellular M. bovis[46]. Under our investigation, TLR2 was significantly upregulated in MAP infected cattle. This is in concurrence with earlier reports which demonstrate that TLR2 expression is upregulated in bovine monocytes following *M. avium* subsp. *paratuberculosis* infection in vitro[47] and also in subclinical and clinical forms of sheep *paratuberculosis*[48]. Similarly, TLR2 gene was found to be differentially expressed (upregulated) in the infected MDM at the 2 hrs post MAP infection[28]. In case of IL2 gene, our analysis revealed significant down regulation of IL2 in PBMC of MAP infected cattle as compared to uninfected healthy control cattle. In agreement with our findings, downregulation of the proinflammatory cytokines IL2 and TNFα were observed in ovine MDMs infected with the selected Map isolates[37]. It indicates a reduced proinflammatory immune response mediated by IL2 and TNFα-2 in infected cells. It is well known that the expression of these cytokines in the presence of intracellular bacteria is one of the first steps leading to activation of

macrophages and effective bacteria killing. In the present study, expression of IL8 was found to be significantly upregulated in PBMC's of MAP infected cattle. Similar to our findings, the expression of IL-8 was significantly ($P < 0.02$) up-regulated in MDM cells infected *invitro* with MAP, relative to uninfected control cells[49]. However non significant differences were observed for IL-8 expression when peripheral blood from cattle with subclinical MAP infection was stimulated with *M. avium* subsp. *paratuberculosis* antigens than that from uninfected cattle[49]. No significant difference in IL16 and TLR4 gene expression was observed between MAP infected cattle and healthy controls. The primers and conditions optimized in the present study were sensitive, fast and reliable in the detection and quantification of TLRs and cytokine mRNAs, and the GAPDH gene was demonstrated to be an ideal internal control gene in bovine experiments. Such real-time PCR assays can be used in Bos indicus cattle to investigate the bovine immune response in a variety of situations, such as viral, bacterial and parasite infections. Findings in present investigation validate the differential expression of key innate immune response genes in naturally infected cattle.

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