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mRNA Expression of Chemokine Genes in Bovine Tuberculosis Infected Crossbred Cattle

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Host genetic variation in disease resistance is mainly attributable to variability in host immune responses to infection. Analysis of variation in host gene expression following M. bovis exposure and infection in cattle offers scope for a better understanding of the molecular regulation of the immune response. Present investigation was conducted to analyze expression profiles of chemokines CXCR3 and CCL1 using qRT-PCR in M. bovis infected cattle and healthy controls and to assess whether any inherent gene expression patterns are visible for these key innate immune response genes. Distinctive gene expression patterns were observed between M. bovis infected cattle and healthy controls. Expression of CXCR3 gene was significantly upregulated (5.22 fold, p=0.0225) in PBMCs of M. bovis infected cattle vis a vis healthy controls. CXCR3 and its ligands are associated with inflammatory diseases of importance to livestock as well as with protective immunity to infectious diseases and tumors. However for CCL1 gene, non significant differences in gene expression were observed between in macrophages of M bovis infected cattle and healthy controls. Distinctive mRNA expression pattern offer potential for developing biomarkers for diseases in livestock.

Keywords: Expression, M bovis, Genes, Immune response, Cattle.

Bovine Tuberculosis (bTB) is chronic disease caused by acid fast bacterium *Mycobacterium bovis (M. bovis*). It has been included in the "List B" diseases of Office Internationale des Epizooties (OIE) (OIE 2008). The disease results in serious costs to farming economies, as well as a danger to human health. *M. bovis* is the aetiological agent of these infections, and belongs to the *M. tuberculosis* complex, which consists of a group of very closely related species of controversial classification[1]. Identification of bovine tuberculosis in live animals mainly depends on clinical manifestations of the disease, skin testing, and subsequent identification of the pathogen by biochemical testing[2]. Most widely used diagnostic method for bTB is based on intradermal tests which are based on a measurable cellular immune response against *M. bovis* that is elicited during the first stages of the disease. In countries implementing BTB eradication programmes, routine screening of herds to detect the presence of animals exposed to *M. bovis* is based on intradermal tuberculin testing. A positive test is generally followed by animal movement restrictions and compulsory slaughter of test-positive animals in an attempt to remove the disease from the herd. Single intradermal comparative cervical tuberculin test (SICCT), uti-lizing *M. bovis* purified derivative protein (PPDB) and *Mycobacterium avium* PPD (PPDA) has a higher specificity by recognizing cross-reactive responses

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against environmental mycobacteria[3,4,5] and allows adjustment for final interpretation. An enzyme-linked immuno-sorbent assay performed on whole blood and detects cell-mediated release of IFN-gamma in response to incubation with PPD-B[6]. The Gamma-Interferon assay (IFN) is also based on the cell-immune response and measures IFN released into whole-blood culture [7] *in vitro*, in response to specific antigen stimula-tion. This assay has been evaluated as a primary diagnostic method in many countries[8] and has some important advantages[5]. The Gamma-Interferon assay (IFN) is also based on the cell-immune response and measures IFN released into whole-blood culture 7 *in vitro*, in response to specific antigen stimula-tion. This assay has been evaluated as a primary diagnostic method in many countries 8 including Brazil, and has some important advantages[5]. Since lymphocyte stimulation is done *in vitro*, and conse-quently does not alter the immune status of the animal, it is not necessary to wait 60-90 days to repeat the test when the initial test is inconclusive, a distinct limitation of the skin tests. Though BTB infection has a worldwide presence, it has been reported to be endemic in Africa and Indian sub-continent[9]. In many developing countries with inadequate surveillance and control activities zoonotic bovine TB is present. Pathogenesis of bovine tuberculosis begins with bacterial entry to host lungs by inhalation and bacteria phagocytosis by alveolar macrophages. Establishment of a chronic infection status is accomplished due to mycobacterial virulence factors that allow it to enter and survive within the host phagocytic cells. It is well known that macrophages play an important role in tuberculosis pathogenesis, being the first defense line, the niche for the bacteria and the main control mechanism[10]. The bactericidal efficacy of macrophage is considered the basis of innate immunity to tuberculosis[11,12] and a phenotypic marker of cattle resistance to *M. bovis* infection[13]. It is believed that the host innate immunity provides the initial resistance to infections with intracellular pathogens before the adaptive type 1 cell-mediated immunity fully develops. The major cellular components involved in innate immunity include phagocytes, macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) cells, γδ T cells, and soluble mediators released by these cells serve as a linker to cell-mediated immunity[14] Effective immune responses are believed to primarily rely on CMI or TH1 responses that involve macrophages, dendritic cells and an adaptive T cell response. These responses are controlled by cytokines released from antigen-specific T cells. Protective immunity to tuberculosis is dependent on the cooperative action of antigen-specific T cells and macrophages, wherein the macrophages ultimately control infection by inhibiting growth of the phagocytosed mycobacteria[15]. Cytokines secreted by the mycobacteria-specific T cells are required for the induction of potent anti-microbial activity. Comparison of mRNA expression allows the identification of genes that are differentially expressed, or in response to certain treatments. gene expression signatures can be recognized in a host's response to a specific pathogen. This may be ascribed to pathogen-driven differences in reprogramming of host gene transcription at the host pathogen interaction level. Genes, coding for proteins with very specific and unique roles in immune responses are potential strong candidates for investigating genetic basis of disease resistance. Chemokine receptor 3 (CXCR-3) gene product is involved in chemotactic T-cell migration, dendritic cell maturation and recruitment of inflammatory cells during infections[16]. Chemokine (C-C motif) ligand 1 (CCL1) gene encodes a cytokine that displays chemotactic activity for monocytes[17] and associated with a proinflammatory immune response.

Gene expression studies of host responses to infection can provide a powerful tool for understanding the interactions between pathogens and the host immune system and may be particularly powerful in identifying specific molecules or pathways that have been targeted by pathogens for immune evasion[14]. Investigation of gene transcriptional changes *in vitro* provides a model for understanding the complex host-pathogen interactions and innate cellular signalling pathways that underlie the host immune response to mycobacterial infection. Genes, coding for proteins with very specific and unique roles in immune responses are potential strong candidates for investigating genetic basis of disease resistance. 378 gene features were found differentially expressed in bTB infected and control

Holstein Friesian cows, of which 244 were expressed at lower levels (65%) in the infected group[16]. *M. bovis* infection of peripheral blood leukocytes was associated with decreased expression levels of numerous host genes[17]. Significant alterations were observed in expression of genes associated with the inflammatory response and cell signalling pathways[18]. Furthermore, the suppression of immune-associated genes has been detected in vivo in *M. bovis*-infected cattle[19]. The objective of this study was to analyze differential expression profiles of CXCR3 and CCL1 genes in M bovis infected cattle vis a vis uninfected controls.

MATERIALS AND METHODS

Experimental animals

The experimental animals in the study were Frieswal crossbred females raised and maintained under similar environmental conditions at Military Dairy Farm, Bareilly. Experimental animals were tested using Single Intradermal Comparative Cervical Tuberculin (SICCT) and Interferon gamma (IFN-γ) assay to identify bTB positive and negative cattle. 5 ml of Blood was collected from each animal in sterile tubes containing heparin as anticoagulant. For SICCT, 0.1mL of *M. bovis* purified derivative protein (PPDB) was inoculated in the cervical area of each cow along with 0.1mL M. avium purified derivative protein (PPDA) approximately 20cm from the PPDB inoculation in the cervical area. After 72 h, the site of inoculation was measured with calipers, and the cow was considered negative if the difference between the thicknesses of both sites of inoculation were <4.0mm. For IFN-γ assay, heparinized blood samples from the 25 cows were collected for IFN-γ testing. Blood samples were incubated for 24 hrs with 100µl PPDA and 100µl PPDB to stimulate lymphocytes to produce IFN-γ. IFN-γ in the plasma supernatants of each blood aliquot was then determined using a sandwich ELISA. The assay was performed using BOVIGAM® TB Kit according to the manufacturer's instructions (Bovigam, Prionics, Zürich, Switzerland). Apart from tuberculosis, brucellosis and paratuberculosis/Johne's disease are among major intracellular infections that severely affect the cattle population. As similarities exist between the mechanisms of protective immunity against *M. bovis* and against *Brucella abortus* and *M. paratuberculosis*, it is important to take into consideration that some of the gene expression changes observed during *M. bovis* infection may not be specific for *M. bovis* alone. Thus animals selected for expression studies were also screened for brucellosis and paratuberculosis to prevent confounding effect. Three different serological tests viz., rose bengal plate test (RBPT), standard tube agglutination test (STAT) and enzyme linked immunosorbent assay (ELISA) were carried out for testing with Brucellosis. The RBPT and STAT was performed according to prescribed method. The CHEKIT Brucellosis Serum ELISA Test Kit was used to detect antibodies against *B. abortus* in individual serum. Johnin skin test, ELISA (PARACHEK® kit) and Faecal ZN staining were used for testing for Johne's disease. For Johnin skin test, 0.1mL of Johnin PPD antigen was inoculated in the cervical area of each cow alongside PPDA approximately 20cm from the PPDA inoculation. After 72 h, the site of inoculation was measured with calipers, and the cow was considered reactive if the difference between the thicknesses of both sites of inoculation were >4.0mm. Solid phase, indirect enzyme immunoassay was used to detect antibodies to *M. paratuberculosis* in isolated sera using commercial ELISA kit PARACHEK® according to manufacturer's instructions. For Faecal ZN staining, 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. Eight animals diagnosed as positive for bTB but negative for Brucellosis and paratuberculosis and eight animals diagnosed as negative for bTB, Brucellosis and paratuberculosis were taken for further gene expression studies.

Peripheral Blood Mononuclear Cells (PBMC) isolation

20 ml of Blood was collected from six positive and six negative cattle in sterile tubes containing heparin as anticoagulant and kept on ice until it was transferred to the laboratory. 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. PBMCs were incubated and cultured in RPMI-1640 media supplemented with 15% Foetal Bovine Serum (FBS) and 100 µg/ml streptomycin at 37 °C in 5% CO_2 and differentiate monocytes for 5-9 days into macrophages with replacement of media 2-3times during the differentiation period

Total RNA extraction and cDNA synthesis

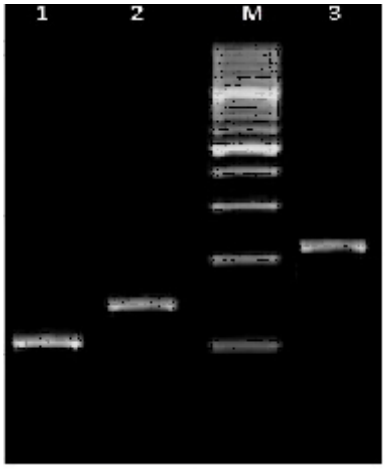
RNA was extracted from the cells using Trizol reagent (Invitrogen, USA), according to manufacturer's instructions and treated with DNase using RNAfree DNase treatment (Thermo Scientific, USA). The quality and quantity of the RNA was determined by gel electrophoresis and Nanodrop spectrophotometer at 260 and 280 nm wavelengths. First strand cDNA was reverse transcribed from approximately 2 µg total RNA using oligo (dT) primer with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) in a final volume of 20 µl according to the manufacturer's instruction. cDNA samples were stored at -20^{0}C until use.

Quantitative reverse transcription-PCR (qRT-PCR) analysis

Gene specific primers for CXCR3 and CCL1 were used for real time expression profiling (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference

Table 1. Real Time PCR primers for the selected gene fragments of Bos taurus

Gene Name	Chromosome No.	Sequence (5' -3')	Amplicon size (bp)
CXCR3	2	F: GAAAGCAGTGTGGACATAGCCA R: CGGAACTTGACACCCACAAAG	101
CCL1	19	F: AGGCTGGATCTGCTCCCAAAT R: GGTGATGTGTGCAAGTTCACCA	152
GAPDH	5	F: CTCCCAACGTGTCTGTTGTG R: TGAGCTTGACAAAGTGGTCG	222



Lane 1 : CXCR-3 (101 bp), Lane 2: CCL1 (152 bp); Lane 3: GAPDH (222 bp) ; Lane M : 100 bp marker

Fig. 1. PCR amplification of chemokines and GAPDH in Macrophages

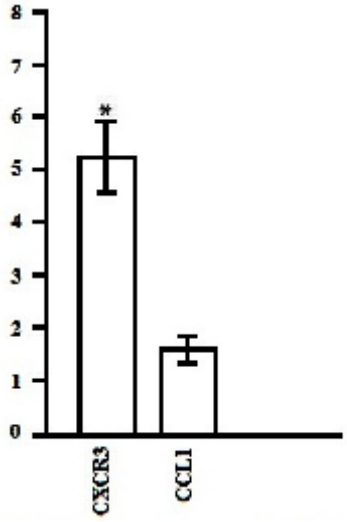


Fig. 2. Difference in expression (fold change values) in macrophages of M.bovis infected cattle as compared to healthy controls. Error bars show standard error of mean of each gne. *P ≤0.05

gene. Maxima SYBR Green qPCR Master Mix (2X) was used for PCR amplification in a final volume of 25 µl. The mix was optimized for efficient and reproducible PCR. A negative control without template (cDNA) was always kept in order to check any PCR carryover. The amplified products were observed under UV transilluminator and documented under Gel Documentation system. Cycling conditions for qRT-PCR assay were 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and 58°C for 30 s and 72°C for 30 s. The specificity of a single amplicon was verified by dissociation curve analysis. Each PCR experiment was carried out in triplicates. 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 specificity of qRT-PCR products was further confirmed by gel electrophoresis. 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[24]. Relative expression of each sample was calculated using the $2^{-\Delta\Delta CT}$ method[23]. Normalization was done for expression of target genes to the expression of reference gene. Student's *t* test was used for testing significance of data. Three technical replicates were averaged and the results were reported as Mean ± SE. Results were analysed and shown as Fold change ($2^{-\Delta\Delta CT}$).

RESULTS AND DISCUSSION

CXCR3 and CCL1 genes selected for the study were amplified from cDNA of macrophages (Fig. 1) to check the specificity of the primers. CXCR3 gene displayed differential expression between macrophages of M bovis infected cattle and healthy controls (Fig.2). mRNA expression of CXCR3 gene was significantly upregulated (5.22 fold, p=0.0225) in macrophages of M bovis infected cattle as compared to healthy controls. However for CCL1 gene, elevated (1.5 fold) but non-significant differences in gene expression were observed between in macrophages of M bovis infected cattle and healthy controls. In the current study, we compared the gene expression profile induced in macrophages of M bovis infected cattle with that of control. 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. Several workers have previously reported that the immunospecific gene expression undergoes shutoff in *M. bovis*-infected cattle. Expression of several genes involved in the innate immune response was suppressed in *M. bovis*-infected cattle[18]. In addition, two consecutive studies of the same research group have reported that the balance in the transcriptional changes induced by *M. bovis* in different cell blood populations is suppression of gene expression[18, 19]. Dissecting variation in gene expression between infected and healthy controls for the genes involved in pathways of immune response to bTB 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. Quantitative Real Time 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[20]. 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[21]. Genes included under study were based on relevant literature in cattle and other ruminants. Here, existence of distinctive gene expression patterns were observed between PBMC of healthy cattle and those exposed to M.bovis. The primers and conditions optimized in the present study were sensitive, fast and reliable in the detection and quantification of cytokine mRNAs, and the GAPDH gene was demonstrated to be an ideal internal control gene in bovine experiments.

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