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Differential Expression Profile of Innate Immune Response Genes Between Indigenous and Crossbred Cattle

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Genetic variation has been observed for susceptibility/ resistance to several infectious diseases in cattle. Superiority of *Bos indicus* breeds over *Bos taurus* breeds for disease resistance trait has been reported by several workers. Therefore the objective of this study was to analyze expression profiles of genes with well characterized roles in immune response using qRT-PCR in indigenous Tharparkar and crossbred Vrindavani cattle and to assess whether significant variation exists in gene expression between two breeds of cattle. Eighteen genes in two groups with GAPDH as reference were investigated for mRNA expression. Ten out of eighteen genes under investigation namely TLR2, NFKB1, TNF, IFNG, IL2, CXCR3, PRKCB1, RPS6KB2, STK17B and EEF1 genes revealed significantly higher expression in indigenous Tharparkar cattle as compared to Vrindavani cattle. Whereas, no significant difference was observed for expression of IL16, CCL1, EEF1G, CD84, MCL1, NFATC4 and IER-5 genes between Tharparkar and Vrindavani cattle. Expression of CD-81 gene was significantly lower in Tharparkar as compared to Vrindavani. Investigation revealed higher basal expression in majority of innate response genes under study in Tharparkar as compared to Vrindavani indicating a superior level of basal immune competence in Tharparkar. This can be a possible reason for differences in susceptibility/resistance in indigenous and crossbreds to various invading bacterial and viral pathogens.

Key words: Resistance, Expression, Tharparkar, Immune Response, Genes.

Selection in dairy animals has been practiced for milk production traits alone for several decades leading to detrimental effect on animal health and welfare. Infectious diseases have major negative effects on livestock production, both in terms of economics and on animal welfare. 10-25 % loss in milk production efficiency has been reported in tuberculosis infected animals[1,2]. Annual economic loss incurred by dairy industry in India on account of udder infections was estimated to be Rs. 6053.21 crores[3]. 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[4]. While traditional control measures such as vaccination, treatment and eradication exist, improving the genetic resistance to diseases among livestock populations is an effective alternate strategy as genetic gain is cumulative and permanent. Estimates of the heritability for susceptibility to Bovine Tuberculosis (BTB), MAP infection, Somatic Cell Count (SCC) were 0.18, 0.16 and 0.11 respectively[5,6,7] demonstrating genetic variation for susceptibility to various infections in dairy cattle.

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Between-breed differences have also been reported for several infectious diseases in livestock. Prevalence of bovine tuberculosis and severity of its pathology was reported higher in *Bos taurus* and crossbreds as compared to *Bos indicus* cattle[8]. Macrophages from Nellore breed were found more efficient to control Brucella abortus intracellular survival than Holstein[9]. Sahiwal calves were more resistant than Holstein dairy breed calves to tick-borne tropical theileriosis[10]. These findings indicate the superiority of indigenous *Bos indicus* breeds over *Bos taurus* breeds for disease resistance trait.

Genes, coding for proteins with very specific and unique roles in immune responses are potential strong candidates for investigating genetic basis of disease resistance. Toll-like Receptor-2 (TLR-2) gene encodes key pathogen recognition receptor involved in activation of the innate immune response and development of adaptive immune responses[11]. It is implicated in the immune response against *M. bovis*, MAP, *Brucella abortus* and other bacteria[12,13]. Activation of TLR is known to result in the activation of the Nuclear factor kappa beta (NFKB) signaling pathway, and finally, results in the release of pro-inflammatory cytokines[14,15]. Tumor Necrosis Factor (TNF) gene codes for a key inflammatory cytokine activated by NFKB transcription factor signaling controls the activation of AP1 downstream signaling pathways[16]. Chemokine receptor 3 (CXCR-3) gene product is involved in chemotactic T-cell migration, dendritic cell maturation and recruitment of inflammatory cells during infections[17]. Chemokine (C-C motif) ligand 1 (CCL1) gene encodes a cytokine that displays chemotactic activity for monocytes[18] and associated with a proinflammatory immune response. Interferon Gamma (IFNG) and Interleukin-2 (IL-2) gene codes for proinflammatory cytokines which are important component of T cell response generated following the initial exposure to intracellular pathogens mainly *M. bovis* and MAP[19,20]. Interleukin-16 (IL-16) gene encodes a potent chemotactic cytokine for CD4+ T cells, has both pro-inflammatory and immunoregulatory properties[21]. Cluster of Differentiation 84 (CD-84) and CD-81 gene express on mature B-cells, T-cell subsets, monocytes and macrophage enhances T-cell activation and cytokine production in infections. Genes encoding adaptor and mediator molecules of the various key immune related signalling activation pathway viz. Ribosomal Protein S6 Kinase-2 (RPS6KB2), Serine/ Threonine Kinase 17b (STK17B), Nuclear factor of activated T-cells (NFATC), Eukaryotic Translation Elongation Factor 1 Gamma (EEF1G), Immediate early response 5 (IER-5), Eukaryotic translation elongation factor 1 (EEF1) gene and Myeloid cell leukemia sequence 1 (MCL1) are also important for modulating and developing effective immune response[14,22,23]. Therefore the objective of this study was to analyze expression profiles of eighteen genes with well characterized roles in innate immune response in indigenous Tharparkar and crossbred Vrindavani cattle.

MATERIALS AND METHODS

Ethical Approval

Blood samples used in this study were collected from Indian Veterinary Research Institute (IVRI) farm after the approval of Institutional Animal Ethics Committee.

Animals

Twelve adult Tharparkar females and twelve adult Vrindavani females were included in the study. All 24 animals were apparently healthy with no recent disease records and all animals were raised and maintained under similar environmental conditions at Cattle & Buffalo Farm, IVRI, Izatnagar. 5 ml of Blood was collected from each animal in sterile tubes containing heparin as anticoagulant.

Isolation of Peripheral blood mononuclear cells (PBMC) in Tharparkar cattle

PBMC were isolated from fresh heparinized whole 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). Cell viability was determined by Trypan Blue staining.

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 (ΔT) 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

18 gene specific primers (TLR2, NFKB1, IL2, IL16, TNF, IFNG, CXCR3, CCL1, CD81, CD84, RPS6KB2, STK17B, PRKCB1, MCL1, EEF1G, NFATC4, EEF1 and IER5) were used for real time expression profiling[24] (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene. Maxima SYBR Green qPCR Master Mix (2X) was used for PCR amplification in a final

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

Gene Name	Chromosome No.	Sequence (5' -3')	Amplicon size (bp)
TLR2	17	F: CCATTGACAAGAAGGCCAT R: AACCCTTCCTGCTGAGTCTCAT	107
NFKB1	6	F: ATACTGAACAATGCCTTCCGG R: CACGTCAATGGCCTCAGTGTAG	135
IL2	17	F: CTTGCACTCGTTGCAAACG R: CAAGCTCTCCAGGATGCATACA	183
IL16	21	F: CGCGGTTTGAAGAATGGAAC R: TCACAGGTCCATCAGGCAAC	51
TNF	23	F: TCTACCAGGGAGGAGTCTTCCA R: GTCCGGCAGGTTGATCTCA	68
IFNG	5	F: TGATGGCATGTCAGACAGCA R: GGCACAAGTCATATAGCCTGACAC	51
CXCR3	2	F: GAAAGCAGTGTGGACATAGCCA R: CGGAACTTGACACCCACAAAG	101
CCL1	19	F: AGGCTGGATCTGCTCCCAAAT R: GGTGATGTGTGCAAGTTCACCA	152
CD81	29	F: TTCATGTCCTGAAGCTCCCTGT R: TGAAGGCATAAGGCTGCTCGT	284
CD84	3	F: TAAGTGGTGTGTCATGGCAGGT R: GGCTGGAGGCTGAATATGACTG	103
RPS6KB2	17	F: TGTGGAACTGGCCTATGCCTTC R: AAGATGCCTTCTCGCTCCAGGT	105
STK17B	2	F: ACAGGCCCTCTTGTAATGGCAC R: AGCAAATCGGACACAAGCTCG	136
PRKCB1	25	F: ATCGAGAGGGAGGTCCTCAT R: GGTCTTGGTCTTCTGCTTGC	141
MCL1	3	F: AGGTGACTGAAAGGCCTGTCTC R: CAACATGTGCCTCTTCTCCCT	244
EEF1G	29	F: TGGATGCTCACTTGAAGACG R: ACTGGGCCATTTTCTCACAG	222
NFATC4	24	F: AACCACTGCCCCTCTCTGAAAC R: CCTCGACCCCAGATCACAAAGA	107
EEF1	21	F: TGGATGCTCACTTGAAGACG R: ACTGGGCCATTTTCTCACAG	222
IER5	16	F: AAGACCCCCGAGACTTCG R: ACACTCTTCAAGGCGGAGAG	115
GAPDH	5	F: CTCCCAACGTGTCTGTTGTG R: TGAGCTTGACAAAGTGGTCG	222

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. The qRT-PCR assay was performed using the CFX Real-Time PCR Detection Systems (BioRad, USA). For, EEF1, IER5, IL2, IL16, EEF1G, PRKCB1, TLR2, NFKB1and TNF genes, annealing temperature was 58°C while for CD81, CD84, RPS6KB2, STK17B, CXCR3, MCL1, CCL1, IFNG and NFATC4 annealing temperature of 60°C was used. Cycling conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and 58/60°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 ($2^{-\Delta\Delta CT}$).

Table 2. Significant Fold Change differences in gene expression in Tharparkar relative to Vrindavani breed

Gene	Tharparkar vs. Vrindavani Relative Expression	P-value
TLR2	1.92 ± 0.16	0.0325
NFKB1	4.66 ± 0.48	0.0193
TNF	1.90 ± 0.01	0.0001
IFNG	6.03 ± 0.64	0.0163
IL2	3.38 ± 0.56	0.0369
CXCR3	2.56 ± 0.34	0.0193
STK17B	2.44 ± 0.25	0.0487
CD81	-0.41 ± 0.02	0.0007
RPS6KB2	2.56 ± 0.17	0.0184
EEF1	267 ± 0.26	0.0121
PRKCB1	8.27 ± 1.33	0.0428

RESULTS

PCR amplification of selected immune response genes in PBMCs

Immune response genes selected for the study (TLR2, NFKB1, IL2, IL16, TNF, IFNG, CXCR3, CCL1, CD81, CD84, RPS6KB2, STK17B, PRKCB1, MCL1, EEF1G, NFATC4, EEF1 and IER5) were amplified from cDNA of PBMCs (Fig. 1a & 1b) to check the specificity of the primers.

qRT-PCR expression profile in Vrindavani and Tharparkar cattle

Eleven out of eighteen genes under investigation displayed differential expression between Vrindavani and Tharparkar cattle (Fig.2). Ten genes (TLR2, NFKB1, TNF, IFNG, IL2, CXCR3, PRKCB1, RPS6KB2, STK17B and EEF1) were found significantly upregulated in PBMC's of indigenous Tharparkar cattle as compared to Vrindavani (Table 2). Highest fold change expression was found in PRKCB1 gene (8.27 fold,

Fig. 1a. PCR amplification of selected genes at annealing temperature 58°C
Lane 1: TLR2 (107 bp); Lane 2: NFKB1 (135 bp); Lane 3: IER5 (115 bp); Lane 4: PRKCB1 (141 bp); Lane 5: HBP-1 (212 bp); Lane 6: IL16 (51 bp); Lane 7: IL2 (183 bp); Lane 8: TNF (98 bp); Lane 9: HBF1-G (222 bp); M: 100 bp ladder

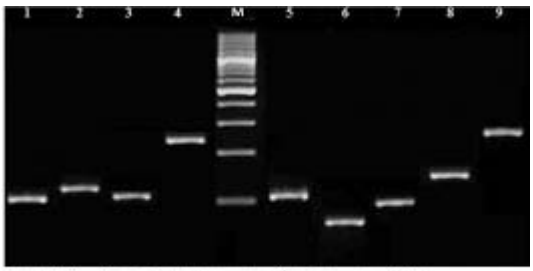

Fig. 1b. PCR amplification of selected genes at amplification temperature 60°C
Lane 1: CD84 (103 bp); Lane 2: STK17B (136 bp); Lane 3: RPS6KB2 (105 bp); Lane 4: MCL1 (244 bp); Lane 5: NFATC (107 bp); Lane 6: IFNG (51 bp); Lane 7 : CXCR3 (101); Lane 8: CCL1(152 bp); Lane 9: CD81 (284 bp) M: 100 bp ladder

p=0.0428) followed by IFNG gene (6.03 fold, p=0.0163) in Tharparkar as compared to crossbreds. Expression of CD-81 gene was significantly downregulated (0.42 fold, p=0.0007) in PBMC's of Tharparkar as compared to Vrindavani. Seven genes (IL16, CCL1, EEF1G, CD84, MCL1, NFATC4 and IER-5) did not reveal any significant difference between Tharparkar and Vrindavani. Although the mRNA expression was elevated in CD-84, NFATC4 and IER-5, the increase was not found to be statistically significant.

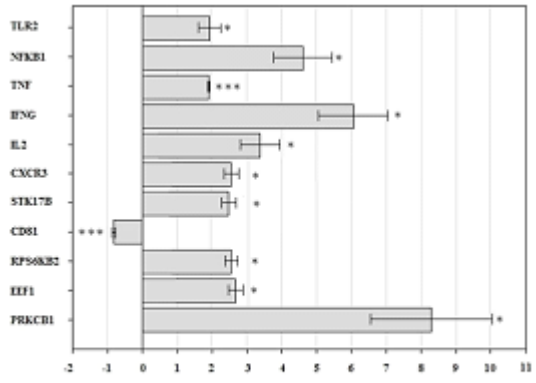

Fig. 2 Differential gene expression between Vrindavani and Tharparkar cattle using q RT-PCR. Fold Change values are shown for PBMC from Tharparkar (n=8) cattle relative to PBMC from Vrindavani (n=8) cattle. Error Bars show standard error of mean of each gene. Statistical significance of each gene is as follows: *** P ≤ 0.001; * P ≤ 0.05

DISCUSSION

Between-breed differences have been shown to exist against several infectious diseases in livestock[8,9,10]. Host genetic variation in disease resistance is mainly attributable to variability in host immune responses to infection[27]. At the cellular level, the interaction between the host and pathogen involves changes in gene expression[28]. Dissecting variation in gene expression between indigenous and crossbred cattle breeds for the key immunity genes will allow us to test the hypothesis that variation in expression of these genes influences the immune status of the breeds. Hence we focused on genes which have been previously reported to be associated with susceptibility/ resistance against several livestock diseases. In the present study, an effort has been made to analyze the differential expression for the selected eighteen genes involved in immune response between indigenous Tharparkar and crossbred Vrindavani cattle using Quantitative Real Time PCR (qRT-PCR). qRT-PCR offer the ability to survey changes in gene expression and has been widely used to discern patterns of host gene regulation during infection[29]. qRT-PCR can accurately detect even low abundance mRNAs, does not require post-PCR processing and functions over a large dynamic range of starting cDNA quantities making it method of choice for accurately quantifying gene expression levels[30]. With the extended panel of 8 cattle per study group, the statistical significance of the mean altered gene expression within each group of cattle could be assessed with greater accuracy.

In the present investigation, 10 genes under investigation (TLR2, NFKB1, TNF, IFNG, IL2, CXCR3, PRKCB1, RPS6KB2, STK17B and EEF1) displayed significantly higher expression in indigenous Tharparkar as compared to Vrindavani cattle. These results indicates a higher level of basal generalized immune competence in the indigenous breed which may provide them with protection/tolerance to combat the various infections better as compared to crossbreds. TLR2, NFKB1, CXCR3, PRKCB1, STK17B, CD81 and RPS6KB2 genes have been previously reported to be repressed in BTB infected cattle as compared to healthy cattle controls suggesting that innate immune signalling is decreased or repressed in chronic BTB-infected cattle as compared to normal healthy cattle[24]. Our findings indicates an agreement with previous reports on differences in Bos taurus and Bos indicus for disease resistance/ immune reponse traits[8,9,10]. Our results indicate higher TLR-2 expression levels may be protective for indigenous breed. Polymorphisms in TLR2 gene have also been reported to be significantly associated with somatic cell score and paratuberculosis infection in cattle[31,32]. NFKB1 gene, central mediator of the proinflammatory immune response was expressed at significantly reduced levels in PMBC of BTB-infected animals and was hypothesized as key mediator of the gene repression detected in BTB infected group[24]. Human and bovine promoter sequences of the differentially expressed genes showed over-representation for the binding sites of NFKB transcription factor in bovine cells infected with FMD disease virus indicating that NFKB is key mediator of several signalling pathways governing immune response against FMD virus[33].

Suppressed immune gene expression of TNF has been demonstrated as a key feature of late-stage M. bovis infection in bovine lymph node[34]. Expression of TNF and its receptors closely associated with disease progression in sheep experimentally infected with Bovine Leukemia Virus (BLV) and TNF mRNA expression was significantly up-regulated in BLV-resistant sheep[35]. It was however found upregulated in BTB infected animals[24]. Through its capacity to activate macrophages and initiate induction of other cytokines, IFNG was postulated to play a significant role in controlling mycobacterial[36]. Animals in the excretory subclinical stage of MAP infection are reported to have increased IFNG expression locally at the site of infection[37]and higher IFNG production in culture supernatants after stimulation of PBMC with MAP antigens[38]. Decreased IL-2, TNF and IFNG levels were observed during bovine ostertagiosis and after challenge[39]. The expressions of IFN-gamma and IL2 mRNAs were significantly reduced in the PBMCs from bovine leukemia virus - persistent lymphocytosis infected cows as compared to BLV negative cows[40]. SNP in IL2 gene showed association with resistance against gastrointestinal infection by nematodes[41]. CXCR3 and its ligands are associated with inflammatory diseases of importance to livestock as well as with protective immunity to infectious diseases and tumors[42]. It was expressed at significantly lower levels in BTB-infected animals[24]. PRKCB1, RPS6KB2 and STK17B genes were found significantly downregulated in BTB-infected animals[24]. CD-81 gene codes for molecule involved in Class I MHC mediated antigen processing and presentation and B Cell Receptor Signaling Pathway. No significant difference in IL16, CCL1, EEF1G, CD84, MCL1, NFATC4 and IER-5 gene expression between Tharparkar and Vrindavani. However CCL1, MCL1 & IER-5 genes were reported to be significantly downregulated in BTB- infected cattle[24]. Whereas EEF1G, CD84 and NFATC4 were significantly upregulated in BTB- infected cattle[24]. Majority of genes under present investigation revealed significant higher expression in indigenous Bos indicus breed suggesting that differential expression may contribute of superiority over Bos taurus or crossbreds for immune response traits.

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