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Research paper

Global gene expression profile of peripheral blood mononuclear cells challenged with *Theileria annulata* in crossbred and indigenous cattle

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ABSTRACT

Bovine tropical theileriosis is an important haemoprotozoan disease associated with high rates of morbidity and mortality particularly in exotic and crossbred cattle. It is one of the major constraints of the livestock development programmes in India and Southeast Asia. Indigenous cattle (*Bos indicus*) are reported to be comparatively less affected than exotic and crossbred cattle. However, genetic basis of resistance to tropical theileriosis in indigenous cattle is not well documented. Recent studies incited an idea that differentially expressed genes in exotic and indigenous cattle play significant role in breed specific resistance to tropical theileriosis. The present study was designed to determine the global gene expression profile in peripheral blood mononuclear cells derived from indigenous (Tharparkar) and cross-bred cattle following in vitro infection of *T. annulata* (Parbhani strain). Two separate microarray experiments were carried out each for cross-bred and Tharparkar cattle. The crossbred cattle showed 1082 differentially expressed genes (DEGs). Out of total DEGs, 597 genes were down-regulated and 485 were up-regulated. Their fold change varied from 2283.93 to −4816.02. Tharparkar cattle showed 875 differentially expressed genes including 451 down-regulated and 424 up-regulated. The fold change varied from 94.93 to −19.20. A subset of genes was validated by qRT-PCR and results were correlated well with microarray data indicating that microarray results provided an accurate report of transcript level. Functional annotation study of DEGs confirmed their involvement in various pathways including response to oxidative stress, immune system regulation, cell proliferation, cytoskeletal changes, kinases activity and apoptosis. Gene network analysis of these DEGs plays an important role to understand the interaction among genes. It is therefore, hypothesized that the different susceptibility to tropical theileriosis exhibited by indigenous and crossbred cattle is due to breed-specific differences in the dealing of infected cells with other immune cells, which ultimately influence the immune response responded against *T. annulata* infection.

1. Introduction

Globally bovine tropical theileriosis is one of the tick-borne diseases with great economic impact on livestock production due to high morbidity and mortality (Jensen et al., 2008). The disease is more important in exotic cattle that are introduced into disease endemic areas to enhance production, thus posing a major constraint on breed improvement programmes of cattle farming in several developing countries including India, where the estimated cost of tropical theileriosis was determined at US$ 384.3 million (Minjauw and McLeod, 2003). In India, several reports ranging from subclinical infection to severe outbreak due to *T. annulata* have been documented (Bansal et al., 1987; Shastri et al., 1980). The prevalence of tropical theileriosis in crossbred cattle has been reported from Bangalore (Ananda et al., 2009), Kerala (Nair et al., 2011), Uttarakhand (Kohli et al., 2014a, b), Odisha (Panda et al., 2011) and Punjab (Shahnawaz et al., 2011). Earlier (1988–1999) records from Gujarat and Andhra Pradesh indicate outbreaks of tropical theileriosis in exotic and crossbred cattle (Kohli et al., 2014a, b). Whereas incidence in indigenous cattle breeds is uncommon (Venkatasubramanian and Rao, 1993).

The causative protozoan, *Theileria annulata* is transmitted principally by *Hyalomma anatolicum*, which has a wide geographical distribution from the Mediterranean basin to China. After infection with *T. annulata*, disease leads to potentially fatal pathology in susceptible animals,

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although long-term protection occurs in recovered, treated or vaccinated cattle (Pipano, 1995). The infectious sporozoite stage of the parasite exhibits cell tropism, predominantly to invading bovine macrophages and to a lesser extent to B lymphocytes (Glass et al., 1989). The parasite has potential to cause substantial phenotypic alterations to its host cells including continuous proliferation, cytoskeletal modifications and resistance to apoptosis or cell death (Kinnaird et al., 2013). Due to lymphoproliferative nature of the disease, infected host cells undergo uncontrolled proliferation following differentiation of the parasite into macroschizont stage (Irvin and Morrison, 1987). Moreover, the parasite evades host response that protects against uncontrolled leukocyte proliferation by regulating the pathways that control programmed cell death (Dessauge et al., 2005b; Heussler et al., 1999). Theileria cell division is coupled to host cell cytokinesis by tight association with the leukocyte mitotic apparatus with the parasite being physically pulled apart as daughter leukocytes separate (Hulliger et al., 1965). Other phenotypic changes to the infected host cell includes, a loss of cellular differentiation markers and changed expression of host genes encoding cytokines, proteases, adhesion and surface receptors molecules (Ahmed and Mehlhorn, 1999; Dobbelaere and Heussler, 1999).

Currently a control strategy for theileriosis is focused on vector control by repeated application of chemical acaricides and use of *anti*-parasite drugs. Repeated and indiscriminate use of these chemicals has led top emergence of resistance in the vectors against the chemical acaricides (Shyma et al., 2012). Besides, attenuated cell culture based vaccines are available but require organized and coordinated vaccination programmes. The best approach to control tropical theileriosis, like other tick-borne diseases, includes a portfolio of integrated strategies that are economically and environmentally sustainable. Use of pre-existing genetically resistant cattle breeds like Kenana *(Bos taurus)* from Sudan and Sahiwal *(Bos indicus)* from Punjab in India, which have been experimentally shown to be relatively resistant to tropical theileriosis may be a suitable alternative (Bakheit and Latif, 2002; Glass et al., 2005). *Theileria* parasite has the ability to generate widespread change to host cell gene expression in a complex and multifactorial manner. Large number of differentially expressed genes (DEGs), which may play an important role in the breed-specific resistance of indigenous cattle to tropical theileriosis, have been revealed in bovine macrophage-specific (BoMP) cDNA microarray (Jensen et al., 2006). With the advancement of information in genomics, new opportunities to identify genes controlling disease resistance can be further explored (Soller and Andersson, 1998).

Therefore, the present study was undertaken to investigate the transcriptome profiles of *Theileria annulata* infected PBMCs of crossbred (called Vrindavani cattle, has the exotic inheritance of Holstein-Friesian, Brown Swiss, Jersey and indigenous inheritance of Hariana cattle (Singh et al., 2011)) and indigenous (Tharparkar) cattle in order to identify DEGs along with their interaction among themselves and various dysregulated functional pathways.

2. Materials and methods

2.1. Strain and experimental animals

T. annulata Parbhani strain originally isolated from Maharashtra (India) and maintained as cryopreserved stabilates of ground-up tick tissue sporozoite (GUTS) of infected *H. anatolicum anatolicum* in the Protozoology laboratory of the Division of Parasitology of the institute was used in the present study. The Ground up tick supernatant (GUTS) was prepared following the protocol of Pipano et al. (2008) with minor modifications. The ticks collected from ear bags of infected calves, were counted and washed with distilled water followed by sterilized with 70% alcohol. The ticks were placed in mortar along with 10 ml of serum free media RPMI-1640 media, triturated very carefully with pestle until all the parts of the ticks were fully macerated and then 3.4% of BSA (bovine serum albumin) was added. The suspension was transferred to 100 ml measuring cylinder and left it for 30 min to settle down the debris. The clear supernatant was transferred to 50 ml tube and 0.1 volume of DMSO (dimethyl sulphaoxide) was added as cryoprotecting agent and mixed properly. The GUTS were initially stored at −70 °C for 6–8 h, with applying cotton on both side of vials for slow freezing and then transferred to liquid nitrogen (LN2) for storage.

The infectivity of cryopreserved stabilates was checked in vivo in crossbred calves. Two crossbred (*Bos taurus* × *Bos indicus*) and two Tharparkar bovine calves, aged 3–4 months, were used in the present study. The calves were checked for general health condition and screened for haemoprotozoan infections by blood smears examination. Animal experimentations were conducted as per the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and Institutional Animal Ethics Committee (IAEC).

2.2. PBMC isolation and in vitro challenge with T. annulata sporozoites

Peripheral blood mononuclear cells were isolated from aseptically collected blood under cold conditions by density gradient centrifugation (Histopaque-1.083, Sigma) (Jensen et al., 2006). Thereafter, one half of each PBMC sample was infected with *T. annulata* (Parbhani strain) sporozoite preparations, as described (Jensen et al., 2008). In short, the separated cells were resuspended at 2×10^6 cells/ml in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS) and aliquoted into a six-well plate. An equal volume of sporozoite suspension at 0.5 tick equivalent/ml in RPMI-1640 medium having 40% FBS was added to the PBMC and incubated at 37 °C in a 5% CO_2 for 2 h. Sporozoite infection in PBMC was confirmed by PCR reaction using *T. annulata* specific small subunit ribosomal RNA (SSU rRNA) gene primers (D'Oliveira et al., 1995). *Theileria annulata* piroplasmic DNA was used as positive control in PCR reaction. The PCR amplification resulted in amplicon of ~372 bp size (Supplementary Fig. 1).

2.3. RNA extraction and analysis

After 2 h of incubation, PBMC culture was harvested from plates in 15 ml centrifuge tubes and supernatant was removed after centrifugation at 1500 rpm for 10 min. RNA isolation was done by using RNeasy Plus Mini Kit (Qiagen) as per the instructions supplied with kit with minor modifications. The RNA was quantified by NanoDrop ND 1000 Spectrophotometer (Thermo Scientific, USA) and integrity of RNA was determined by 2100 Bioanalyzer (Agilent Technologies, USA). The samples having RIN value >8.0 were selected for further experiment.

2.4. Microarray experimentation and analysis

Two separate microarray experiments were carried out using Bovine (V2) Gene Expression Microarray, 4x44K(Agilent). Two biological replicate samples were profiled per condition (i.e. replicate samples each in crossbred and Tharparkar cattle). A 200 ng of total RNA was used to prepare Cyanine-3 (Cy3) labeled cRNA for hybridization. One-Color Low input Quick Amp labeling Kit (Agilent) was used for labeling followed by cleaning using RNeasy column purification kit (Qiagen). Dye integration and cRNA concentration were determined with the NanoDrop ND-1000 Spectrophotometer. For chip hybridization, 1.65 μg of Cy3 labeled cRNAs was fragmented at 60 °C for 30 min in a reaction volume of 55 μl containing 25 × Agilent Fragmentation buffer and 10 × Agilent Gene Expression Blocking agent. On completion, 55 μl of 2 × HI-RPM hybridization buffer (Agilent) was added. Out of total volume, 100 μl of samples were hybridized on Bovine (V2) Gene Expression Microarray 4x44K for 17 h at 65 °C in a rotating hybridization chamber (Agilent). Following hybridization, microarray slides were washed with Gene Expression wash buffer 1 (Agilent) for 1 min at room temperature and with Gene Expression wash buffer 2 (prewarmed at 37 °C for overnight) at 37 °C for 1 min. The slides were scanned with an Agilent SureScan Microarray Scanner. The scanned images were analysed with Agilent Feature

Extraction Software 11.0.1. Data generated were analysed by GeneSpring software version 13.0. Paired *t*-test ($p \leq 0.05$, logFold change (FC) ≥ 1.5) was used to find differentially expressed genes. After bioinformatic analysis of generated microarray data, many probes present on microarray slide did not show the gene symbols. To overcome this problem, the probes (60 mer) which did not show gene symbols were checked in NCBI blast and the respective gene symbol were included in the further study.

2.5. Gene ontology (GO) and protein-protein interaction network analysis

Functional enrichment analysis of DEGs in different pathways was done using database for annotation, visualization and integrated discovery (DAVID, v6.7) (Huang et al. 2007). All significant pathways ($p \leq 0.05$), enriched in database were considered for further study. Protein-protein interactions were determined for *Bos taurus* orthologs of human using Biological General Repository for Interaction Datasets (BioGRID) and visualized in Cytoscape 3.1.1 (Shannon et al., 2003).

2.6. Validation of microarray results by qRT-PCR

Microarray results for seven genes (Table 1) that are either involved in *Theileria* infection or showed breed specific differences in their expression were verified using qRT-PCR. Four biological and three technical replicates were used in validation study for each breed. These four samples include the two samples that were used for the microarray experiment for each breed. The cDNA synthesis was carried out using 500 ng total RNA and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems,UK) in 20 μl reaction with random primers according to manufacturer's instructions. The product was thereafter stored at −20 °C prior to perform real time quantitative reverse transcription PCR (qRT-PCR). For qRT-PCR, designed primers (Table 1) and Fast SYBR® Green Master mix (Applied Biosystems,UK) were used on a 7500 Fast Real-Time PCR System apparatus (Applied Biosystems, UK). The qPCR thermal cycling programme consisted of one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. A dissociation step was included to confirm amplification specificity. Data generated were analysed by comparative C_T method (Schmittgen and Livak, 2008) and paired *t*-test.

3. Results

3.1. Identification of DEGs in T. annulata infected crossbred and Tharparkar PBMC

On analysis, 1082 and 875 genes were found to be differentially expressed in *T. annulata* infected crossbred and Tharparkar PBMCs, respectively. Out of total DEGs, 597 and 451 genes were found to be downregulated, and 485 and 424 genes were upregulated in infected crossbred and Tharparkar PBMC, respectively. The fold change varied from 2283.93 to −4816.02 and from 94.93 to −19.20 in crossbred and Tharparkar cattle respectively (Supplementary Table 1). The common differentially expressed genes between crossbred and Tharparkar cattle were 71 (Fig. 1). The expression data generated from the current study were submitted in the NCBI Gene Expression Omnibus (GEO) repository (GEO accession number- GSE81419) (Barrett et al., 2011).

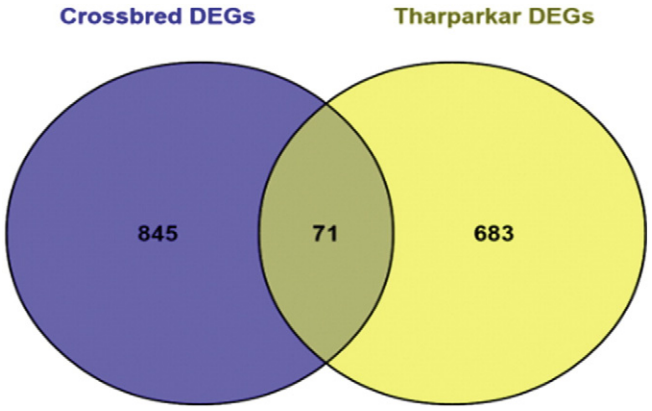

Fig. 1. Common differentially regulated genes (DEGs) between Crossbred and Tharparkar cattle.

3.2. Functional annotation of DEGs in crossbred and Tharparkar cattle

Using DAVID, GO terms for 49 biological processes, 10 cellular components and 29 molecular functions were identified ($p < 0.05$) in crossbred cattle. The highest fold enriched GO terms in biological process included positive regulation of foam cell differentiation, cellular response to oxidative stress, leucocyte mediated cytotoxicity, response to oxidative stress, antigen processing and presentation of peptide etc. (Fig. 2a). The highest fold enriched GO terms in cellular component included MHC class II protein complex, soluble fraction, MHC protein complex etc. (Fig. 2b). In molecular functions, 3′-5′ cyclic nucleotide phosphodiesterase, protein dimerization and homodimerization activity and receptor binding etc.(Fig. 2c) were the highest folds enriched GO terms. The list of GO terms involved in biological processes, cellular components and molecular functions are mentioned in Supplementary Table 2.

In Tharparkar cattle, GO terms for 112 biological processes, 55 cellular components and 9 molecular functions were identified

Table 1
List of genes under investigation with their log fold change for validation of microarray data by qRT-PCR.

Genes	Primer sequence	Product Size	Log fold change by qRT-PCR	Log fold change by microarray analysis
NFKBID	F AATCAGCACCACGGAATCCCC R TCTTCTAGCAGCTTCTTCACCG	82 bp	−0.88	−0.83
IL1RAP	F GGGACTTCTGTGGTGTGTAATG R TGGATCTGCCTCATGGTATCT	105 bp	−1.05	1.41
BoLA-DQB	F GGCTCTAAACCACCACAACC R GGAACCACCGAACCTTGAT	77 bp	−1.33	−1.96
HOXA13	F GAAGCGTGTCCCTTACACCA R CTCCGAGAGATTCGTCGTGG	118 bp	0.87	1.15
PAK1	F GCCAAGCCTTCTGTGAAATAAA R GAGAGCACTAGACCAGGAAATG	95 bp	0.57	1.22
TGFBR2	F GTTCAAGTGGACTGAGAGAGATTAG R AGCTATTGGGACAGCATCAC	101 bp	−2.8	−0.91
NFKBIA	F AGAACCTTCAGACACTGCCG R ATCGTAGGGAAGCTCGTCCT	86 bp	−0.82	−0.65
GAPDH	F GGCGTGAACCACGAGAAGTATAA R CCCTCCACGATGCCAAAGT	194 bp		

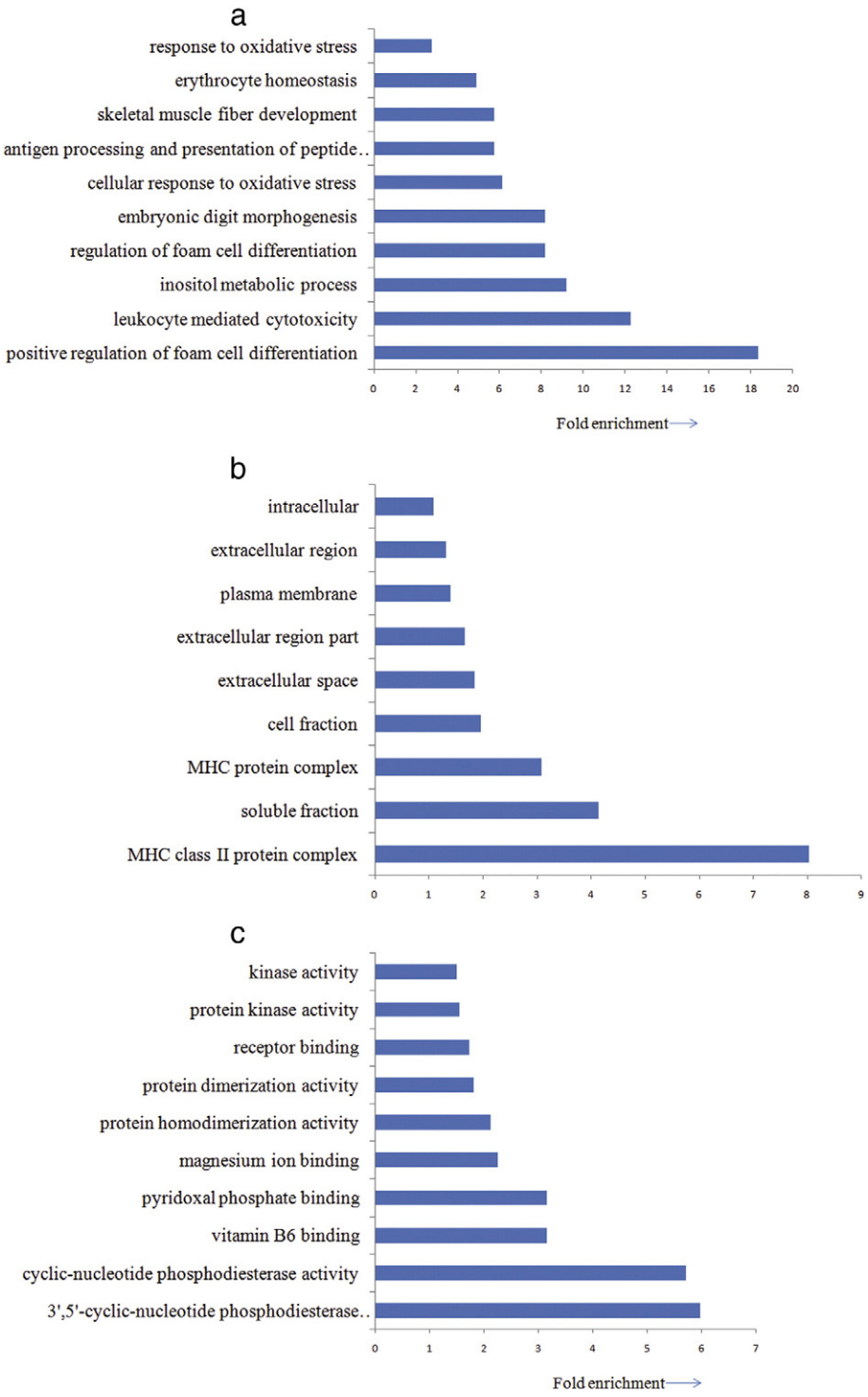

Fig. 2. a Highest fold enriched GO terms of Biological processes in crossbred cattle ($p < 0.05$). 2b Highest fold enriched GO terms of cellular components in crossbred cattle ($p < 0.05$). 2c Highest fold enriched GO terms of Molecular functions in crossbred cattle ($p < 0.05$).

(Supplementary Table 2). The highest fold enriched GO terms in biological process included immunoglobulin secretion, response to peptidoglycan, regulation of membrane protein, mitotic spindle organization, regulation of NF-κB import into nucleus etc. (Fig. 3a). The highest fold enriched GO terms in cellular component includes spindle microtubule, trans-golgi network, spindle etc. (Fig. 3b). In molecular functions, microtubule binding motor protein, non-motor actin binding protein, actin binding cytoskeleton protein and G protein modulator etc.(Fig. 3c) were highest fold enriched GO terms.

3.3. Protein-protein interaction network among the differentially expressed genes

As the protein-protein interactions in *Bos taurus* were not well defined in BIOGRID database, human protein-protein interactions were used to find out the interactions of the all DEGs among themselves. In case of crossbred cattle, all DEGs were used to check gene interaction among themselves. After removing self duplicates and duplicate edges, 238 nodes and 308 edges were identified. These genes were

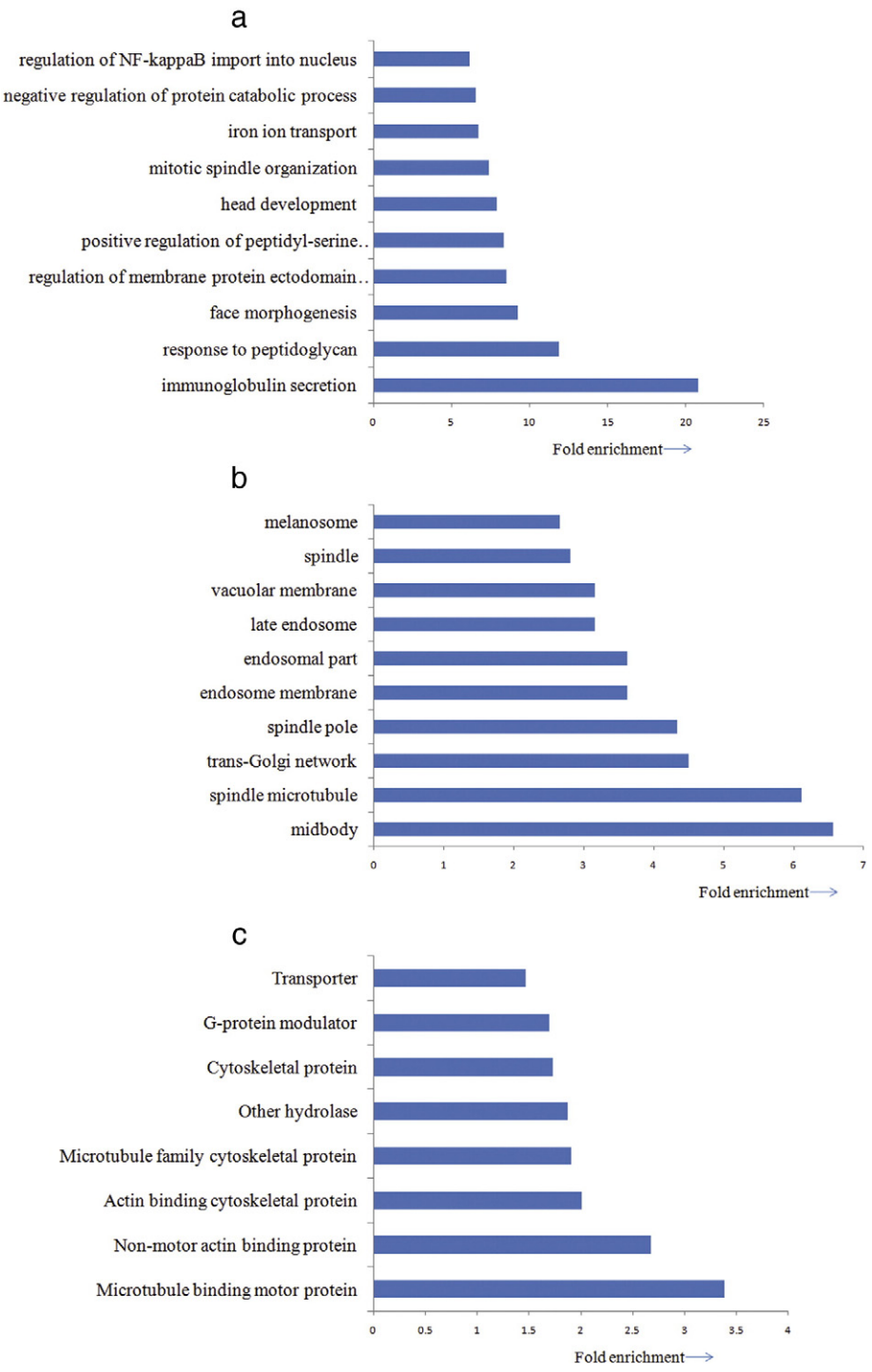

Fig. 3. a Highest fold enriched GO terms of Biological processes in Tharparkar cattle ($p < 0.05$). 3b Highest fold enriched GO terms of Molecular functions in Tharparkar cattle ($p < 0.05$). 3c Highest fold enriched GO terms of cellular components in Tharparkar cattle ($p < 0.05$).

designated as Differentially Expressed Highly Connected (DEHC) genes (Fig. 4). Similarly in Tharparkar cattle also, after removing self duplicates and duplicate edges, 114 nodes and 129 edges were identified (Fig. 5).

3.4. Validation of microarray data by qRT-PCR

The log fold change results of microarray and qRT-PCR were compared for NFKBID, BoLA-DQB, IL1RAP, HOXA13, TGFBR2, NFKBIA and PAK1genes (Table 1, Fig. 6). The change in expression of all the genes except for IL1RAP gene was in concordance with microarray result indicating that the microarray results provide an accurate report of transcript levels.

4. Discussion

Despite of many advances over the last few decades in ameliorating the impact of tropical theileriosis on livestock species, the control of disease still remains one of the most important global priorities for effective livestock management. In recent years, focus has been shifted to functional genomics technologies that interrogate the host transcriptome in response to *T. annulata* infection. In particular, microarray

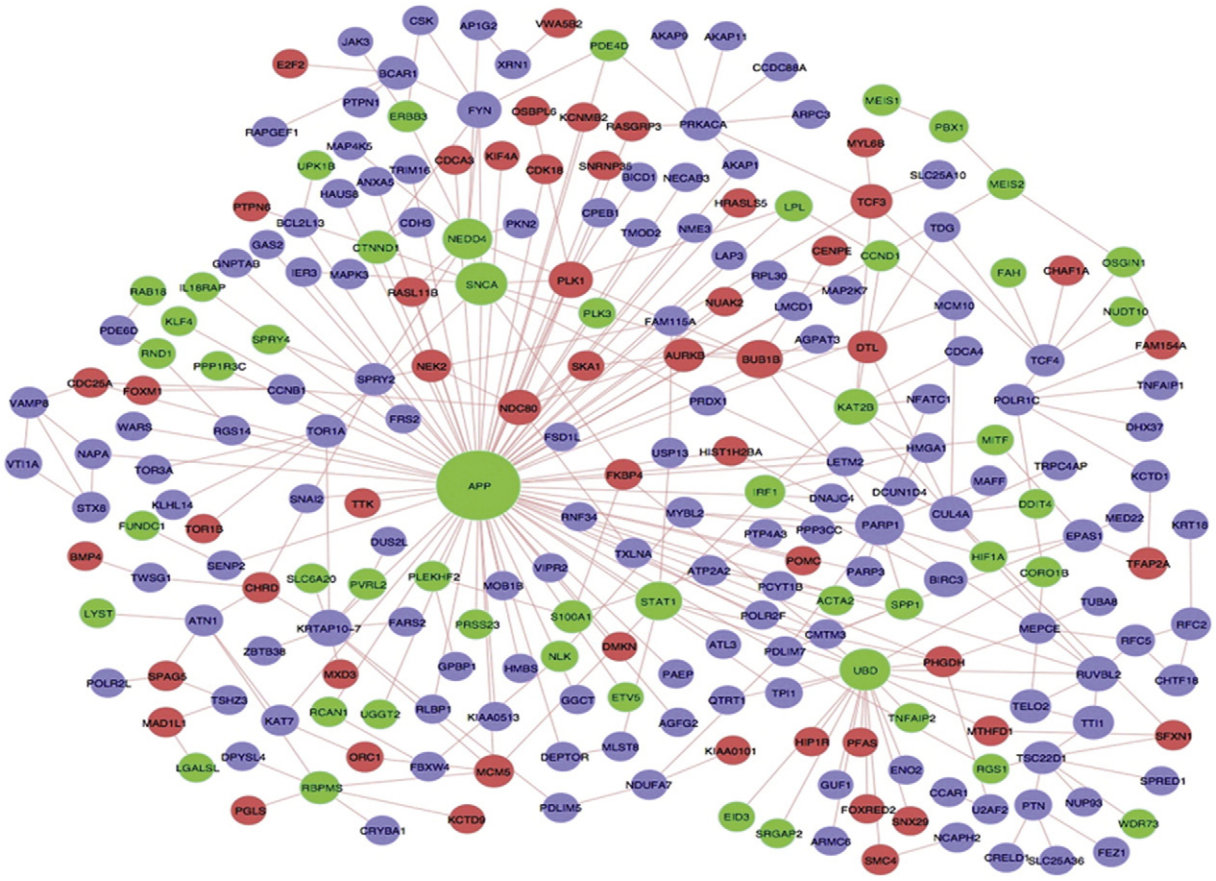

Fig. 4. Protein-protein interaction network among DEGs in crossbred cattle. Green colour is showing upregulation (Fold change > 2), red colour is showing downregulation (Fold change > 2) and blue colour is showing both up and downregulation (Fold change < 2).

technology along with rapid development of more sophisticated bovine genome resources has permits high-resolution analyses of genes and pathways governing the host response to infection with *T. annulata*. Previously, transcriptional differences in in vitro *T. annulata* challenged monocytes from tolerant (Sahiwal, *Bos indicus*) and susceptible (Holstein–Friesian, *Bos taurus*) cattle breeds have been studied using bovine macrophage-specific cDNA microarray (Jensen et al., 2008). Another functional genomics approach revealed that the transcriptome profile of the *B. Taurus* macrophages was more coupled with an inflammatory programme than the *B. indicus* macrophages (Glass et al., 2012).

The parasite shows cell tropism predominantly to bovine macrophages and to lesser extent to B lymphocytes. Therefore, using PBMCs in the present study, we compared gene expression profiling of *T. annulata* sporozoite challenged and non-challenged PBMCs, using genome-wide bovine microarray in crossbred and Tharparkar cattle. Microarray experiment provided 1082 DEGs in crossbred and 875 DEGs in Tharparkar cattle ($p < 0.05$, FC > 1.5). Microarray results were validated by SYBR Green based qRT-PCR using GAPDH as reference gene because out of the three constitutive genes (actin, β-tubulin and GAPDH), GAPDH was found to be best suited (Kinnaird et al., 2013). The qRT-PCR outcome correlated well with microarray data indicating that the microarray results provided an accurate report of transcript level. Functional annotation study of differentially expressed genes has confirmed their involvement in various pathways including immune regulation, cell proliferation, cytoskeletal changes and apoptosis which were well established during tropical theileriosis. Network analysis of these differentially expressed genes provided an efficient approach to understand the interaction among them.

Total seven genes (NFKBID, IL1RAP, BoLA-DQB, HOXA13, PAK1, TGFBR2, NFKBIA) that showed breed specific differences or involved in *T. annulata* infection were selected for validation. A strong correlation was found for all genes except IL1RAP which is in agreement with previous studies (Kinnaird et al., 2013; Jensen et al., 2006; Jensen et al., 2008). The IL1RAP gene has been previously shown to be negatively correlated (Dunckley and Lukas, 2003; Le Bouter et al., 2003). Invasive potential of *Theileria*-transformed leukocytes involves TGF-b signaling. Holstein-Friesian cattle which is susceptible to this disease expresses more TGF-b2 and traverse Matrigel as compared to disease-resistant Sahiwal cattle. Thus, TGF-b2 levels correlate with disease susceptibility (Chaussepied et al., 2010). In present experiment, TGFBR2 is downregulated (1.88 fold) in Tharparkar cattle while its expression didn't change in crossbred cattle which may be correlated with less susceptibility of indigenous cattle in *Theileria* infection. TGFα is a potent mitogen for many different cells (Wang et al., 2012) regulates cell proliferation and migration through multiple pathways. TGFA showed upregulation (1.87 fold) in crossbred cattle while downregulated (3.29 fold) in Tharparkar cattle which again gives indication of less susceptibility of Tharparkar cattle.

Out of all DEGs, many genes viz., FCGR1A, SLC11A1, IGHG1, PRSS2, KLK12, and PDLIM1showed wide variation in their expression in both breeds (Tharparkar and crossbred) i.e. upregulation in crossbreds and down-regulation in Tharparkar or vice versa; or very high expression level in crossbreds and very low expression level in Tharparkar or vice versa. These genes exhibited a difference in magnitude of expression (fold change), indicating that the degree of differences in expression is crucial for establishment of parasite in the host and thus may act as critical regulators during early stage of *T. annulata* infection.

FCGR1A gene encodes for high-affinity receptor for Fc region of IgG and plays an important role in both innate and adaptive immune responses including phagocytosis and transduction of stimulatory signals

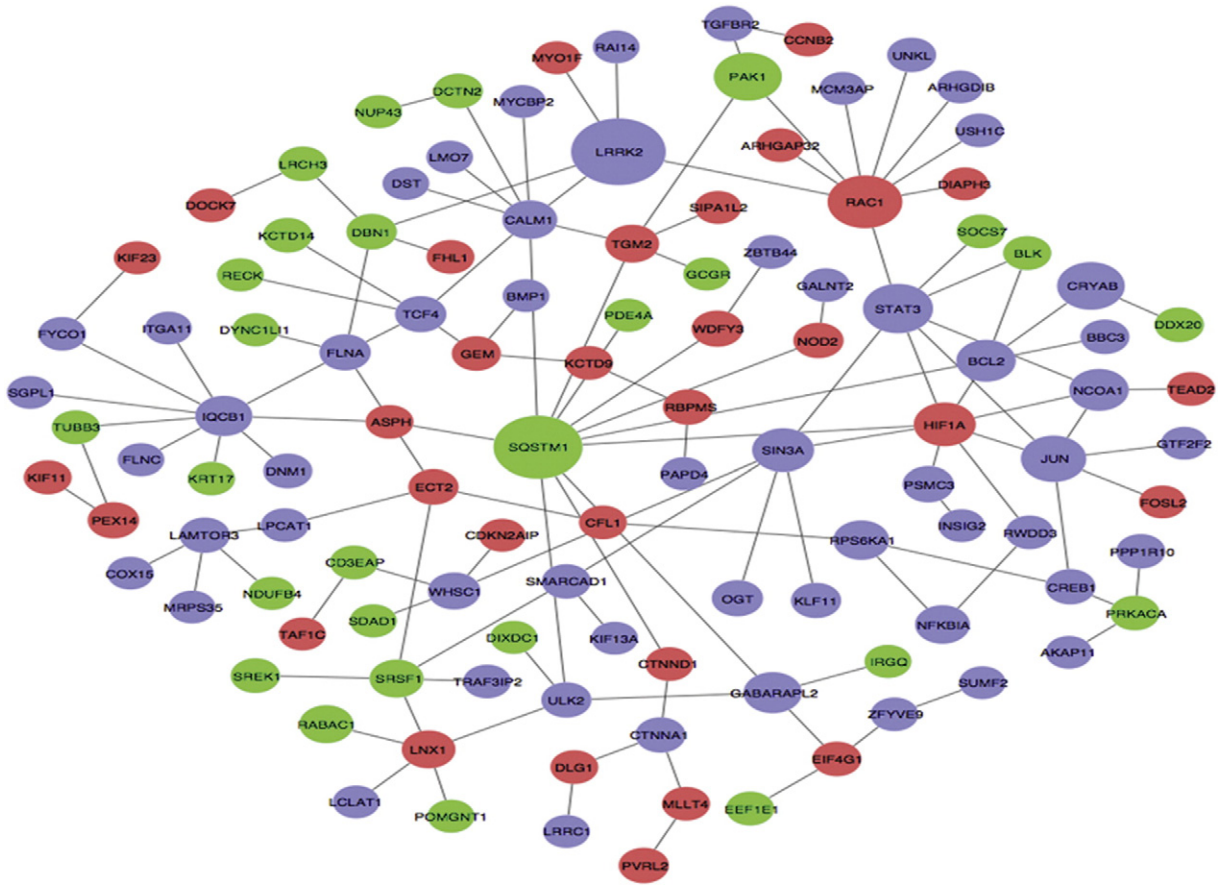

Fig. 5. Protein-protein interaction network among DEGs in Tharparkar cattle. Green colour is showing upregulation (Fold change > 2), red colour is showing downregulation (Fold change > 2) and blue colour is showing both up and downregulation (Fold change < 2).

to monocytes. The GO annotations related to this gene include IgG binding and receptor signaling protein activity (Pander et al., 2010). This gene was found to be upregulated in crossbred (4.54) but downregulated (−2.56) in Tharparkar cattle. The expression difference in this gene might be responsible for high level of pro-inflammatory cytokines in crossbred cattle during *T. annulata* infection. The SLC11A1 gene encodes for macrophage specific natural resistance-associated macrophage protein 1 (Nramp 1) which is a proton-coupled divalent metal ion transporter protein and involved in iron metabolism and natural resistance to infection with intracellular parasites. Pathogen resistance involves sequestration of Fe^{2+} and Mn^{2+} cofactors of both prokaryotic and eukaryotic catalases and superoxide dismutases; not only to protect the macrophage against its own generation of reactive oxygen species, but to deny the cations to the pathogens for synthesis of its protective enzymes. The GO annotations related to this gene include protein homodimerization activity and transition metal ion transmembrane transporter activity (Cellier et al., 1994). The SLC11A1 gene is upregulated in crossbred (2.07) while downregulated (−2.32) in Tharparkar cattle. The expression difference in this gene might be due to host response to high parasitemia in crossbred cattle leading to high pro-inflammatory immune response.

The IGHG1, immunoglobulin heavy constant gamma 1 (G1m marker), a protein-coding gene related to antigen binding is highly downregulated (−52.62) in crossbred cattle while its expression did not differ in Tharparkar cattle. This downregulation may be responsible for poor B-cell adaptive immune response in crossbred cattle. The PRSS2 gene, which is located as a cluster within the T cell receptor beta locus, encodes a trypsinogen, which is a element of the trypsin family of serine proteases. It has been verified that trypsin can act as a signal factor in provoking tumor cells proliferation and destroying matrix to promote cell transfer and as a stimulant of lymphocytes to combat with its inhibitor (α1-antitrypsin, α1-AT/AAT), so to play an important role in tumor immune surveillance (Qing-quan et al., 2013). The PRSS2 showed upregulation in both crossbred and Tharparkar with a very high fold change (2283.93) in crossbred than in Tharparkar (4.59). The PRSS2 converts inactive MMP3 into active MMP3. Members of the matrix metalloproteinase family (MMPs) has been previously found to be highly up-regulated in parasite infected cells (Baylis et al., 1995) and shown to be linked to metastasis of *Theileria* infected cells (Adamson et al., 2000). In our study, crossbred cattle showed 12.74 fold upregulation of MMP1 while Tharparkar cattle showed 2.42 fold upregulation of MMP17 which is in accordance with previous studies (Kinnaird et al., 2013).

The KLK12 gene encodes for kallikrein-related peptidase 12 which belongs to kallikrein subgroup of serine proteases that have diverse physiological functions. The KLK12 gene showed very high upregulation (318.94) in crossbred cattle but its expression in Tharparkar cattle did not differ significantly. Earlier, many kallikreins have been shown as biomarkers in carcinogenesis like over expression of KKL12 in human gastric cancer (Zhao et al., 2012). Upregulation of KLK12 has been demonstrated in mice infected with *Helicobacter pylori*, which is known to be involved in gastric ulcers in human (Toyoda et al., 2013). The PRKACA gene is a signaling molecule, important for a variety of cellular functions. It is downregulated in crossbreds while upregulated in Tharparkar cattle.

DAVID gives a widespread set of functional annotation tools for researchers to get biological meaning behind huge number of genes. Gene ontology terms related to immunological processes, cell proliferation, apoptosis and cytoskeleton organization were found enriched in present experiment. This may due to the fact that following infection

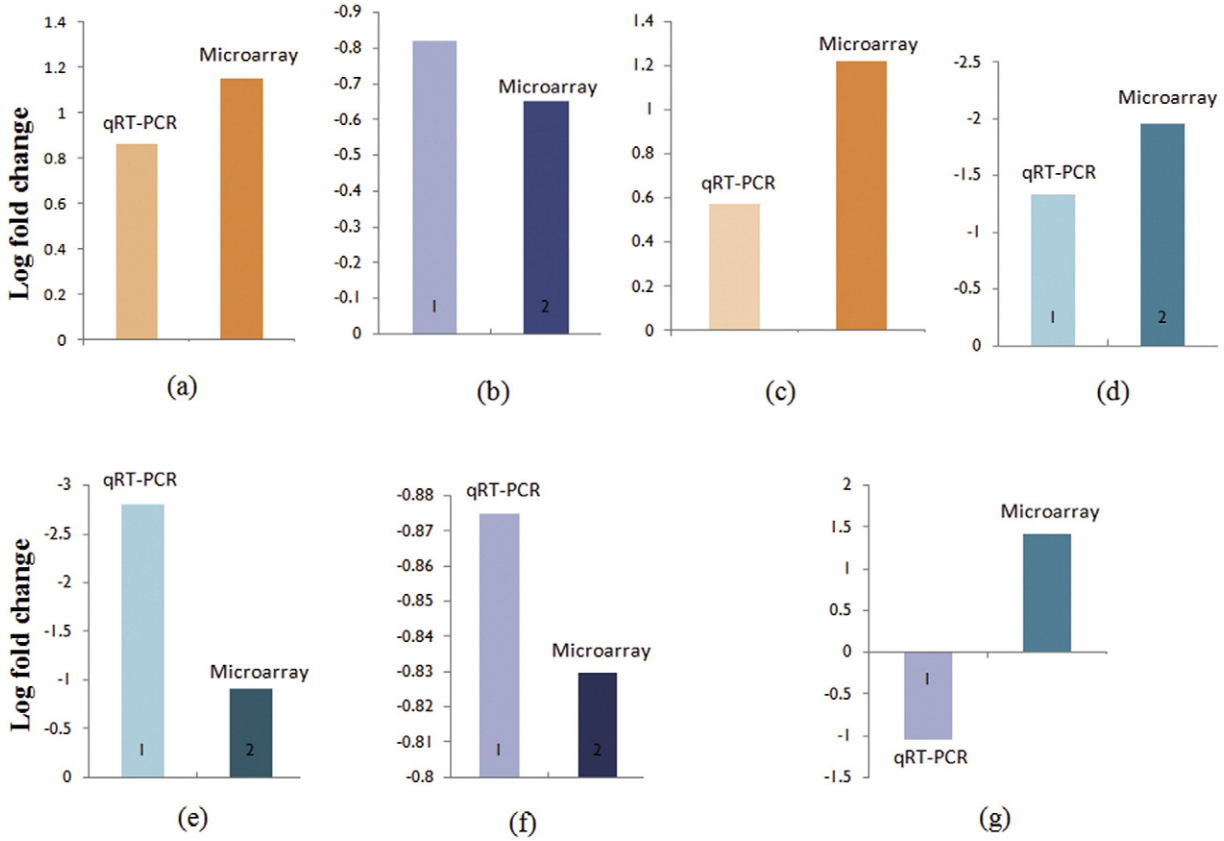

Fig. 6. Direction of log folds change in the selected genes by qRT-PCR and microarray. (a) HOXA13 gene, (b) NFKBIA gene, (c) PAK1 gene, (d) BoLA DQB gene, (e) TGFBR2 gene, (f) NFKBID gene, (g) IL1RAP gene.

of leukocyte; parasite induces expression of key set of host genes that in turn lead to the activation/repression of pathways that are essential for survival/growth of the infected cells. Such genes, which are under direct control of infection, might be predicted. Previous literature also reveals that large number of the differentially expressed genes encode proteins on plasma membrane or in extracellular space and represent one of the major gene ontology biological processes recognized during tropical theileriosis (Jensen et al., 2008). The important pathways represented by the genes are: cell adhesion molecules, cytokine–cytokine receptor interactions and regulation of actin cytoskeleton (Jensen et al., 2008). By pathway analysis, Durrani et al. (2012) identified a set of candidate genes, having role in manipulation of cellular functions, associated with the infected transformed cells. The data indicated that the *T. annulata* parasite can irreversibly reconfigure host cell gene expression networks, having role in development of inflammatory disease and cancer to generate an outcome that is beneficial for survival and propagation of the infected leucocytes.

Protein-protein interaction network (interactome) analysis presents an effective way to understand the interrelationships between the genes (Wachi et al., 2005). BioGRID is a biological database, contains curated protein-protein and genetic interactions for all major model organism species. In this repository, protein-protein interactions in human are well defined with very few for *Bos taurus*. Since protein interactions have been shown to be well-conserved across species (Suthram et al., 2006; Yu et al., 2004), *B. taurus* orthologs in human were extracted for the present study. APP gene had highest (172) interactions with other genes in crossbred cattle while LRRK2 gene had highest (84) interactions with other genes in Tharparkar cattle. Interaction network of crossbred cattle showed many genes which are related to apoptosis and cell proliferation and involve in kinases activity (APP, HIF1A, STAT1, PARP1, MYBL2, BCAR1, FYN, SNCA, SPYR2, UBD, JAK3, CSF2, BMP4, MAP4K1, MAP4K5, etc). Amyloid precursor protein (APP) gene is reported to be associated with cell proliferation and malignancy in humans (Takagi et al., 2013). STAT1 is a member of the signal transducers and activator of transcription family of transcription factors. It is involved in upregulation of genes signaled by either type I, type II or type III interferons. Activation of STAT1 can be pro-apoptotic and considered to be a tumor suppressor (Sironi and Ouchi, 2004). The PARP1 gene is having role in various important cellular processes such as differentiation, proliferation and tumor transformation. All these genes might have direct or indirect role in *T. annulata* pathogenesis.

In Tharparkar cattle, many genes were observed having role in various pathways related to *T. annulata* infection (STAT3, JUN, BCL2, PAK1, TGFBR2, HIF1A, SQSTM1, RAC1, etc). The SQSTM1 gene is involved in regulation of I-kappaB kinase/NF-kappaB cascade pathway and showed very high interaction (74) among differentially expressed genes. The JUN proto-oncogene is a putative transforming gene of avian sarcoma virus 17 and encodes a protein which is very similar to the viral protein and interacts directly with specific target of DNA sequences to regulate gene expression. Previous investigations also reveal that parasite infection leads to an up-regulation of all proteins of members of the Jun./Fos family and surprisingly, this occurs in the absence of any detectable ERK, or p38 MAP kinase activity (Chaussepied et al., 1998). The BCL2 gene encodes an integral outer mitochondrial membrane protein that blocks the apoptosis of some cells such as lymphocytes. The BCL2-related protein *A*1 was significantly upregulated in bovine lymphosarcoma cell line infected with *T. annulata* (Kinnaird et al., 2013). Protein-protein interaction network reveals that HIF1A have well established role in tropical theileriosis. It showed upregulation in crossbred cattle as compared to downregulation in Tharparkar cattle. The activation of this gene is reported to be required for the transformation of leucocyte inside the host system (Medjkane et al., 2014). The PVRL2 protein is one of the plasma membrane components of adherens junctions. This gene is involved in many immune related pathways like regulation of immune

system process, positive regulation of antibody mediated immune response, regulation of immunoglobulin mediated immune response, positive regulation of B cell mediated immunity, positive regulation of lymphocyte or lymphocyte mediated immunity, regulation of adaptive immune response etc. It is downregulated in Tharparkar while upregulated in crossbred cattle.

5. Conclusion

Modulation of the host-gene expression in response to *T. annulata* infection was evident from the large number of differential expressed host-genes between the two experimental groups. Conclusively, the breed specific differences in mRNA of crossbred and Tharparkar cattle may be responsible to susceptibility/resistance to tropical theileriosis at early stage of infection. Although different cattle breeds may have evolved alternative strategies to combat infection and even within Tharparkar, multiple mechanisms may be involved. The common genes which showed expression in opposite direction or large differences in the same direction might be having high impact in disease progress during early stage of infection. The difference in response of both breeds to *T. annulata* infection provides an ideal model to explore the mechanism behind tolerance to tropical theileriosis. Further, detection of genes responsible for resistance would provide a means of getting an environmentally sound and sustainable approach to reduce this economically important disease. However, there is a need to expand the exploration of host–pathogen interactions during *T. annulata* infection at different time intervals for identifying the common biological molecules involved at various stage of infection.

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