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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.

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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

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