Peripheral blood leukocytes transcriptomic signature highlights the altered metabolic pathways by heat stress in zebu cattle

Vamsikrishna Kolli A, R.C. Upadhyay B, Dheer Singh a,b,*

A Molecular Endocrinology Laboratory, Animal Biochemistry Division, National Dairy Research Institute, Karnal 132001, Haryana, India
B Animal Physiology Division, National Dairy Research Institute, Karnal 132001, Haryana, India

Abstract

High temperature during summer greatly affects animal production due to altered reproductive and metabolic functions. However, information regarding high throughput analysis of change in gene expression in dairy animals is relatively nil. In present study, gene expression profiling by microarray was done in peripheral blood leukocytes of heat exposed (42 °C, 4 h) cattle (n = 3), Tharparkar (Bos indicus). A total 460 transcripts were differentially expressed with a fold change of ≥ 2. Randomly selected real-time validation showed that 73.08% correlation with microarray data. Functional annotation and pathway study of the DEGs reveals that, up-regulated genes significantly (P < 0.05) affect the protein processing and NOD like receptor pathways, while down regulated genes were significantly (P < 0.05) found to associated with Glycolytic pathways. In conclusion, the present study showed that heat stress affects expression of significant number of genes in peripheral blood leukocytes and further analysis is required to understand their functional role in livestock.

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1. Introduction

Heat stress is the most stressful abiotic stress to animals including farm animals. In tropical countries like India, summer is the most stressful season for animals because of higher temperatures beyond 42 °C. During this period, animals become more prone to infectious diseases because of decreased immunity. There is also a tremendous loss in animal production due to altered reproductive and metabolic functions. For instance, heat stress (HS) shows negative impacts on all aspects of dairy cattle and buffalo reproduction (Rensis and Scaramuzzi, 2003; Marai and Habeeb, 2010) and production (West, 2003). As an estimate, American Dairy Industry is losing nearly $900 million every year because of heat stress on farm animals (http://www.dairybusiness.com). Similarly, India is losing 2% of the total milk production, which is amounting nearly Rs 2661 crore, due to rise in heat stress on cattle and buffaloes because of the global warming.

Thermal stresses trigger a complex program of gene expression and biochemical adaptive responses (Fujita, 1999; Lindquist, 1986). At the cellular level, it is generally accepted that thermal stress leads to increases in expression of HSPs, which correlates closely with the acquisition of thermo tolerance (Kregel, 2002; Lindquist, 1986; Parsell and Lindquist, 1993). However, it is also increasing apparently that the cellular response to heat shock involves not only HSPs but also several other biomolecules (Kregel, 2002; Sonna et al., 2002). Heat shock has been shown to affect the expression of genes involved in immune function, signal transduction, metabolism, cell growth, proliferation, differentiation etc. (Sonna et al., 2002). Heat stress can have large effects on most aspects of reproductive function in mammals. These include disruptions in oocyte development, oocyte maturation, early embryonic development, foetal and placental growth and lactation. These deleterious effects of heat stress are the result of either the hyperthermia associated with heat stress or the physiological adjustments made by the heat-stressed animal to regulate body temperature (Hansen, 2004). Season of calving significantly affected the milk yield in Indian (Roy Chaudhury and Deskmuykh, 1975) and Egyptian buffaloes (Marai et al., 2009). In Egyptian buffaloes, the highest milk yield was recorded during spring and winter when the calving occurred during the mild period. On contrast, the lowest milk yield was recorded in summer season when the calving occurred during the hot period, (Khashab et al., 1984). Several authors have documented a higher incidence of a variety of intra-mammary infections during summer (Smith et al., 1985; Morse et al., 1988; Waage et al., 1998; Cook et al., 2002). For example, the morbidity rate of Corynebacterium pseudo tuberculosis infection in Israeli dairy cattle was higher during the summer months (Yeruham et al., 2003).

A series of in vitro studies have demonstrated that exposure of bovine peripheral blood mononuclear cells to short and severe heat shocks reduced the responsiveness to mitogens or decrease the number of viable cells (Elvinger et al., 1991). Acute heat shock
altered the gene expression profiling of many heat shock and non-
heat shock proteins in human peripheral blood mononuclear cells
(Sonna et al., 2002). Thus in general, there are numerous reports
suggested that heat stress declines the reproduction, immunity
and all metabolic activities in bovine and other species and causing
huge economical loss to dairy industry (Hansen, 2004; Roy Chau-
dhury and Deskmuykh, 1975; Elvinger et al., 1991; Sonna et al.,
2002). However, there is a lack of information about global expres-
sion profiling for these genes particularly in dairy animals during
heat stress. Hence, studies related to heat stress specifically gov-
erning the global expression profiling of genes involved during
heat stress and further data analysis with various bioinformatics
would provide information regarding affected pathways and met-
abolic functions in cattle.

In the present study, global gene expression profiling in periphe-
ral blood leukocytes of the heat stressed cow (Tharparkar) was per-
formed by using bovine gene chip micro array (~23,000 transcripts).
Additionally, validation of the differentially expressed genes by real
time PCR and Pathway analysis for the effected genes were carried
out to understand the new insights about heat stress effects.

2. Materials and methods

2.1. Animal selection and heat exposure

Three Tharparkar animals were taken for the present study. All
the three animals (n = 3) were exposed to heat stress at 42 °C for
4 h in climatic chamber present in the Institute cattle yard. These
climate chambers have been used for the maintenance of constant
temperature in this experiment as per the Institute’s Animal Ethic
Committee. The chambers are maintained clean and proper care
has been taken for animals with the provision of sufficient water.
During the period of heat exposure, animals were watched by
using the closed circuit cameras. Minimum three animals were
exposed to heat at a time in the available climatic chamber. Blood
samples were collected from jugular vein of the animals before
and after the heat exposure by using the EDTA containing vacu-
tainer tubes. The physiological parameters like rectal temperature,
respiration rate and pulse rate of the animals were also taken to
confirm the heat stress before and after the heat exposure. The col-
clected samples were quickly brought to laboratory for the further
analysis.

2.2. RNA isolation and sample preparation for microarray

RNA was isolated from blood leukocytes by using Ambion leuko-
llock total RNA isolation kit AM1923. The RNA was quantified by
using Nano drop, and RNA integrity was evaluated by denaturing
garose gel electrophoresis. Isolated RNA was used immediately
for RT-PCR or stored at −80 °C until its further use. Samples were
sent to I Life discoveries, Gurgaon, India for microarray analysis.
The RNA purity was measured by Agilent Bioanalyzer within the firm.
RNA samples having RIN value more than 5 were considered as good
quality RNA, and were used for further Microarray analysis. Micro-
array was done by using Affymetrix bovine gene chip, which is a
100-format (Cat log No. 900562), 11 µm array design containing 11
probe pairs per probe set. The design of the array was based on the
content from UniGene and GenBank® mRNAs. The Bovine Genome
Array was developed through the GeneChip® Consortia Program
and it contains 24,027 probe sets representing more than 23,000
transcripts and approximately 19,000 UniGene clusters. Data were
analyzed by GeneSpring software. For validation, the genes com-
monly expressed were selected from all the three samples. List of
the genes and primers used for validation are given in Table 1. A total
of 17 genes were validated, out of that 5 were by absolute quantifi-
cation and remaining was by relative quantification.

2.3. Reverse transcription

cDNA was synthesised by using the First strand cDNA synthesis
kit (Fermentas, St. Leon-Rot, Germany) in a two-step reaction. In
the first step, the reaction mixture contained the following con-
tents: 100 ng of total RNA, 1 µl of random hexamer (0.2 µg/µl)
and dH2O to 11 µl. These contents were incubated at 65 °C for
10 min followed by 2 min incubation at room temperature. In the
second step reaction, the following reagents were further added:
4 µl of 5 × reaction buffer (250 mM Tris–HCl, pH 8.3; 250 mM
KCl, 20 mM MgCl2, 50 mM DTT), 1 µl of RNase inhibitor (20 IU),
2 µl of dNTP mix (10 mM), 2 µl of M-MuLV reverse transcriptase
(200 IU) to a final volume of 20 µl. The experimental conditions
were 25 °C for 10 min, 42 °C for 30 min and 95 °C for 3 min. The
primers were designed by using quant prime and NCBI database.
The PCR was performed and the amplified PCR products were puri-
fied, cloned by Promega TA cloning kit and further sent them for
sequencing.

2.4. Real time PCR – absolute quantification

Absolute quantification has been done by using LC 480 (Roche)
in a 12 µl final reaction mixture containing 100 ng of total RNA
primers, LC 480 SYBR Green master mix (Roche) and dH2O. The
following experimental conditions were used: pre-incubation at 95 °C
for 5 min, followed by 40 cycles of denaturation at 95 °C for 20 s,
aannealing at 60 °C for 15 s, extension at 72 °C for 15 s. Amplification

Table 1

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Gene symbol</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size</th>
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<td>HSF70</td>
<td>CCCCACCTTGAGGAGGTGGTCTG   (24)</td>
<td>AGCCCTTGACCCCAACACGTCG (22)</td>
<td>79</td>
</tr>
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<td>HSF1</td>
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<td>TTGGTAACACTGCTCCTGGGACT (24)</td>
<td>70</td>
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<td>AACTCGTGGGTCGTGCTTCTCTC (22)</td>
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<td>HSPCA</td>
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<td>GAGCTTCGGATGAGTCGACG (22)</td>
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</tr>
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</table>
and quantification had been performed by using light cycler real
time PCR (Roche Diagnostics, Mannheim, Germany). Melting curve
analysis had been done in order to ensure the amplification and
thus the generation of single product. Standard curves were gener-
ated by using the cloned PCR products in the range of over eight or-
ders of magnitude (2.5 \times 10^{-10} \text{ to } 2 \times 10^{-10} \text{ g DNA per reaction}).
Copy number was calculated relative to the amount of RNA that
was subjected to cDNA preparation. PCR reaction with all the PCR
reagents except cDNA was used as a negative control.

2.5. Real time PCR – relative quantification

Relative quantification was done by using Mini optican system
from Bio-Rad. Amplification of cDNA was performed with LC 480
SYBR Green master mix (Roche) in final reaction mixture of
12 µl, and the following cycling conditions were used: pre-incuba-
tion at 95 °C for 5 min, followed by 40 cycles of denaturation at
95 °C for 20 s, annealing at 60 °C for 15 s and extension at 72 °C
for 15 s. Melting curve analysis was done in order to ensure the
amplification and thus generation of a single product. GAPDH gene
had been chosen as an internal control for expression analysis.
Agarose gel (1.5%) electrophoresis was carried out in order to
determine the length of amplified PCR product. All chemicals ex-
cpt cDNA were added for the PCR negative control. \Delta \text{ACT method}
had been used to calculate the relative change in gene expression
among the groups.

2.6. Bioinformatic analyses of microarray data

Gene annotation and functional clustering was performed by
DAVID online database (http://david.abcc.ncifcrf.gov/gene2gen-
e.jsp) for the differentially expressed genes (DEGs). In addition,
Gene ontology and pathway analysis were done by the FunNet on-

2.7. Data analysis

One-way ANOVA was used for the data analysis. The difference
between the treatment means was compared with the use of least
significant difference (LSD) at P < 0.05. All experimental data were
presented as the mean ± SEM of at least three replicates.

3. Results

3.1. Physiological parameters

Rectal temperature, skin temperature and respiration rate were
significantly increased when animals were exposed to heat stress.

Fig. 1. The rectal temperature and skin temperature of different body parts of the animals before and after exposure to heat stress, values are means ± SEM of three
independent animals, one way ANOVA used for statistical analysis. The difference between the means was compared using least significance difference at \( P < 0.05 \).

Fig. 2. Respiration rate of the animals before and after exposure to heat stress, values are means ± SEM of three (\( n = 3 \)) independent animals, One way ANOVA used
for statistical analysis. The difference between the means was compared using least
significance difference at \( P < 0.05 \).

In the present study, above all the three parameters were increased
after exposing to heat stress (Figs. 1 and 2).

3.2. Microarray analysis

Of the three Tharparkar samples analysed by the microarray,
two samples showed more number of commonly expressed
genes and one sample had less number of differentially ex-
pressed genes. So, the first two samples were considered for
the further analysis. However, the genes commonly expressed
in all the three samples were selected for the validation studies.
Among these selected genes, 460 genes showed significantly dif-
ferential expression with a fold change of more than 2. Out of
these 460 genes, 250 genes were found to be down regulated
and 210 genes were up regulated. This differential expression
is depicted in the Heat map (Fig. 3), in which, the expression
change is ranged from red\(^*\) to blue. The red and its allied colours
showed the over expressed genes and the blue and its allied col-
ours showed the under expressed genes. The range of expression
was proportional to the intensities of the colour. Gene clustering
was done from higher expression to lower expression in control
samples; but in the test group the same genes showed the oppo-
site patterns of the expression. Nearly 60 genes identified in the
present study were unnamed and hence their locus numbers have
been considered rather than their annotated names. Similarly,
there were some differentially expressed genes had their gene
IDs but not their reported functions. Interestingly, maximum

\[^{1}\] For interpretation of color in Fig. 3, the reader is referred to the web version of
this article.
(nearly 1/5 of the total genes) numbers of the genes affected by heat stress belonged to transcription and translation related functions. As expected, nearly 26 genes of chaperone and co-chaperone related functions were up regulated during heat stress. For instance, amongst the chaperone related genes, HSP70 (HSPA1A), HSP90 (HSPCA), HSP90B and HSPH1 (HSP10)5 genes were found to be predominantly expressed during heat stress (cited in Supplementary Data).

Results of the present study also demonstrated that a significant numbers of genes belonged to pro apoptosis (BAD, BAK1,BID) and
anti-apoptotic (FBXW12, BCL7C) were shown differential expression. As heat stress is one of the most stressful factor to the animals (Roelofs et al., 2007), signalling mechanisms play a crucial role in the maintenance of stress response by connecting several physiological mechanisms (Martindale and Holbrook, 2002). Genes involved in these signalling pathways were showed differential expression pattern with majority of genes up regulated during heat stress. These results support the fact that when any external stimulation occurs on cells, the cells will communicate with each other and provide signals to all over the body (Cited in Supplementary Table). Another most importing function is the metabolism, heat stress alters the different metabolic functions and pathways, affected genes and their functions were cited in the Supplementary Table and further discussed in the subsequent sections.

3.3. Validation of differential expression

Randomly selected genes related to diverse biological functions identified by using microarray profiling were further validated by real-time PCR (Fig. 4). Significant (**P < 0.05**) differential expression of the selected genes in these validation studies are comparable (73.08% correlation) with the expression pattern obtained by microarray profiling.

3.4. Bioinformatics analyses of microarray data

3.4.1. Gene annotation, functional clustering

By DAVID functional annotation tools, the top five functional clusters were considered for the upregulated genes (Table 2) to understand their role in heat stress. These clusters were selected because of their EASE scores <0.05. The EASE scores are modified Fisher exact P values, which indicate the random assignment of the genes to specific functional cluster. Hence the gene clusters with <0.05 EASE score indicate non-random association of these genes with particular functional clusters. With this background, many upregulated genes during heat stress are involved in leukocyte activation (example: EGR1, IL8), Tetra tricopeptide repeat functions (Example: PPID, SUGT1), Heat shock proteins (HSPH1, HSPA1A, HSPA1B), and ribonucleic acid binding complexes (Example: ACADM, DICER1) (Table 2). Only two functional annotation clusters had genes with <0.05 EASE score for the list of down regulated genes (Table 3). Hence these two clusters were considered for understanding the physiological roles of down regulated genes during heat stress. These two clusters majorly comprising of carbohydrate catabolism genes (TPI1, PKLI, ALDOC, ENO3, BAD) indicated the down regulation of carbohydrate breakdown and energy conservation during heat stress.

3.4.2. Gene ontology and pathway analysis

Gene ontology categories such as biological process, cellular component and molecular functions for up and down regulated genes and their transcriptional domain coverage were shown in Figs. 5–7. The transcriptional domain coverage in the gene ontology categories indicated that the molecular functions of up regulated genes are mainly involved in protein folding and chaperone function, and the down regulated genes are mainly involved in NADH (ubiquinone) dehydrogenase activity, NADH dehydrogenase activity and other metabolic functions. The biological process of up regulated genes mainly covered the positive regulation of cellular and biological process, response to organic and other stresses. The down regulated genes covered the cellular metabolic process, transport and programmed cell death. To find the effected biological pathways in the present study, the pathway (KEGG pathway) analysis (Fig. 8) was performed for the DEGs. The pathways of up
regulated genes mainly emphasized the protein folding in endoplasmic reticulum, NOD like receptor pathway and ubiquitin mediated proteolysis. And the pathway of down regulated genes mainly reinforced the metabolic pathways, Alzheimer’s disease and carbohydrate metabolism like glycolysis, fructose and mannose metabolism, and pentose phosphate pathway.

4. Discussion

In the present study, gene expression profiling was done by Bovine gene chip microarray (~23,000 transcripts, Agilent technologies) in heat stressed peripheral blood leukocytes of the zebu cattle, Tharparkar (Bos indicus). Heat stress causes the major loss in the production and reproduction in the dairy industry (reference). Also diverse physiological and molecular functions (Sonna et al., 2002) of the animals were altered when exposed to high ambient temperatures. Microarray study reveals the differential expression of genes in a particular tissue efficiently in a treatment or diseased condition (Stoughton, 2005). Though the effect of heat stress at global molecular level is comparatively less studied in cattle, so the study on expression profiling of heat exposed cattle could discloses the new facets of this particular scenario.
Physiological parameters play a major role in determining the behavioural changes in cattle during stress conditions (Ganaie et al., 2013). Respiration rate, rectal temperature and body temperature were the physiological markers for the heat stress (Scharf et al., 2011; Ganaie et al., 2013). All these physiological parameters were significantly increased. Respiration rate is increase in heat stress because of the increased blood flow and vasodilation (West, 2003). Rectal temperature and body temperature were increased significantly after exposure to heat stress indicating that animals were under stress after exposure.

The results of microarray reveals ~450 differentially expressed genes of diverse biological functions with >2-fold change. This
indicating the response of the peripheral blood leukocytes to the heat stress in this cattle. Differential expression of these genes will alter the steady functioning of the cells. Up regulation of the HSPs and other co chaperones will help in the acquisition of thermo tolerance and maintain body homeostasis (Kregel, 2002; Lindquist, 1986; Parsell and Lindquist, 1993). In the present study nearly all HSP family proteins were up regulated and this could be the marker for the animals under heat stress and is correlating with the results of physiological parameters. Along with chaperones, genes which related to transcription and translation were also shown up regulation after exposure. Cell signalling is another important function, that coordinates the all the body functions.
during different physiological stimulations (Kholodenko, 2006). Genes involved in these signalling pathways were showed differential expression pattern with majority of genes up regulated during heat stress. These results support the fact that when any external stimulation occurs on cells, the cells will communicate with each other and provide signals to all over the body (Kholodenko, 2006). Summer is the most prone to the mastitis and other infectious diseases in cattle and buffalo (Smith et al., 1985; Morse et al., 1988; Waage et al., 1998; Cook et al., 2002) because of the declined immune response. In the present study most of the immune genes were down regulated after exposure to heat stress. The decreased expression of the immune response may be the decreased lymphocyte count decreased metabolic rate and dry matter intake (DMI), nutrient intake and transport to different tissues. During heat stress metabolic rate and DMI will reduce in cattle (Ganaie et al., 2013). In the present study, expressions of the metabolic genes were down regulated, and predominantly carbohydrate metabolism is down regulating to decrease the internal energy. This might be leads to decreased immune response of the animal during heat stress. Genes involved in these signalling pathways were showed differential expression pattern with majority of genes up regulated.

The Bioinformatics analysis of the microarray data is further supporting the above results. Gene annotation studies of up regulated genes explaining that activation of different cells, HSPs and different nucleotide binding, and down regulated genes mostly covers the clusters belongs to carbohydrate metabolism and other metabolisms. Pathway analysis of up regulated genes covers the proteins processing in endoplasmic reticulum and ubiquitin mediated proteolysis explaining the stress response and down regulation of metabolic pathways chiefly the carbohydrate metabolism showing that decreased energy metabolism of the animal. This possibly will explain that animals were responding to heat stress by up regulating the stress response pathways, and energy metabolism is down regulating to decrease the internal energy. This might be leads to decreased immune response of the animal during the heat stress. Results of gene ontology study explains that heat stress effects the all the biological processes and cellular components and molecular functions of the cells. So, the effect is a global level this will cause the deprivation in the regular biological functions of the animals.

5. Conclusions

In conclusion, the present study explains that heat stress effects on the global gene expression of the cattle. Overall 210 genes were up regulated and 250 genes were down regulated by the heat stress. The gene annotation, gene ontology and pathway analyses for these differentially expressed genes explained that animals were responding to heat stress by increasing the stress regulating genes, and decreasing the expression of energy metabolism genes in order to maintain the body homeostasis. Further studies at functional level for these genes may explain the molecular mechanism involved in this concerned scenario.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.rvsc.2013.11.019.

References


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