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Transcriptome analysis of cattle PBMCs induced with *E. coli* antigen causing mastitis

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Abstract
Endotoxin lipopolysaccharide (LPS) is the most powerful and effective immunostimulant and key virulence factor produced by Escherichia coli (*E. coli*) accountable for many of the inflammatory changes observed during bovine mastitis. In this study, whole-transcriptomic gene changes in bovine peripheral blood mononuclear cells (PBMCs) stimulated with LPS were compared to the control group and analyzed by RNA sequencing. A total of 358 differentially expressed genes (DEGs) were identified in the LPS-induced group as upregulated against the control group, with an adjusted *Padj < 0.05* by edge R. The downstream regulation of the immune system process revealed DEGs were involved in the immune response, cell activation, cytokine-mediated signaling pathway, natural killer cell-mediated immunity, etc. These results offer evidence that LPS antigen activates the early inflammatory response and LPS stimulation significantly alters the expression of genes related to inflammation, immunological control, and antigen processing and presentation in PBMCs.

Keywords: Mastitis, peripheral blood mononuclear cells, lipopolysaccharide, RNA sequencing, escherichia coli

1. Introduction

Bovine mastitis is among the most important dairy animal production diseases, affecting farmers' economies and ultimately, the economy of the nation. India leads the world's milk producers, producing 209.96 million tonnes of milk as an outcome of effective policy decisions during the year 2020-21 as compared to the record of 2019-20 (198.44 million tonnes) with a growth rate of 5.80% (Annual report 2021-22). FAO Food Outlook (November 2022) reported a 0.6% increase in world milk production from 928 million tonnes in 2021 to 930 million tonnes in 2022. Much of the expansion is foreseen to originate in India and Pakistan on rising dairy herds, although at slower growth rates than in earlier years due to animal disease outbreaks and extreme weather events, and in China, sustained by large-scale dairy operations. This represents the slowest rate of growth in the supply of milk and its several products for the world's ever-growing population in the last couple of decades, exacerbating global markets to tighten. In the years 2021-22, milk availability per person has increased to a level of 427 grams per day, which is higher than the global average of around 321 grams per day in 2020 (Ministry of Fisheries, Animal Husbandry & Dairying). In response to shifting food and lifestyle patterns, higher purchasing power and demographic changes, milk consumption is on the rise. However, milk productivity is limited in several developing countries due to poor-quality feed services, diseases, limited market, and service access, and the minimal genetic potential of milk-producing dairy animals (FAO). For an extended period, bovine mastitis has seriously hindered dairy production efficiency. Mastitis incidence in Asia is also rising exponentially including both cattle and buffaloes, posing a significantly challenging task for policymakers, field veterinarians, and researchers. Despite the good management approaches and careful veterinary attention, mastitis persists resulting in significant economic loss all around the world. Genetic control of the production of immune response and resistance to disease in livestock is considered an economic and prophylactic technique for health improvement. Studies have revealed that immunity traits have a moderate-high heritability and have been successfully incorporated into selection programs to produce naturally resistant animals [13]. *Escherichia coli* (*E. coli*) has been identified as among the most common gram-negative bacteria causing clinical mastitis in cattle [14] and LPS which is believed to be a key virulence factor of *E. coli* [15] can evoke powerful responses from the host immune defense playing a primary role in stimulating many transcription factors [10].

The PBMCs which are composed of a wide variety of lymphocytes and monocytes have been commonly employed in comparative pathogenic pathway analysis in cattle disease mechanisms and gene expression levels [12, 5, 7].

To the extend which we believe, comparative expression analysis of the genes linked to LPS-induced bovine mastitis, including the underlying signal transduction cascades, is scarce in crossbred cows like Vrindavani. To fully comprehend the factors impacting the interaction between the causative pathogen and the response of mammary gland cells like PBMCs, we hypothesized in this study that differential gene expression would be reflected in a comparative transcriptomics study comparing LPS-induced PBMCs to controls. Subsequently, explore the functional mechanism associated with immune-mediated pathways. Key genes involved in these pathways could act as candidates for the discovery of polymorphisms to be included in future breeding programs, enabling the selection of naturally resistant animals.

2. Materials and Methods
2.1 Resource population
Three apparently healthy lactating crossbred Vrindavani cattle were identified based on physical examination and with no history of mastitis condition based on the health record maintained at the Cattle and Buffalo Farm, ICAR-Indian Veterinary Research Institute (Uttar Pradesh). Milk samples were scrutinized for the possibility of sub-clinical mastitis using various mastitis-specific assays using two protocols milk somatic cell count (SCC) and the California mastitis test (CMT) score. Thus, five ml blood was collected via the jugular vein of healthy animals (which only included animals with a CMT score of 0 and 1 and SCC less than 200,000) in heparin-coated vacutainers (BD) under sterile circumstances and shaken gently to ensure proper mixing of the blood with the anti-coagulant, thereafter, promptly deposited in an icebox before being transported to the laboratory for subsequent processing.

2.2 PBMCs isolation and stimulation with LPS *in vitro*
PBMCs were extracted through density gradient centrifugation following standard protocols (Histopaque 1077 Sigma-Aldrich). These PBMCs were re-suspended in RPMI-1640 (Sigma-Aldrich, USA) growth media, supplemented with 10% Foetal Calf Serum, and centrifuged for pelleting PBMC. The recovery and viability of the isolated PBMCs were checked by Countess™ Automated Cell Counter (Invitrogen, USA) using 0.4% trypan blue. Viable blood mononuclear cells obtained from each animal were plated at a density of 1×10^6 cells/mL into two rows of the polystyrene 6-well culture plate. One row of culture plates was stimulated with *E. coli* outer membrane antigen LPS (Sigma-Aldrich, Saint Louis, USA) at a dose rate of 1 µg/mL of culture media with a stimulation time of 6 h and another row was kept un-stimulated as a control for this study. The culture plates were kept at 37 ℃ with 5% CO_2 levels.

2.3 Extraction of RNA and RNA sequencing (RNA seq)
Total RNA extraction from the PBMCs was accomplished by the Trizol method (Ambion, USA), as per the guidelines provided by the manufacturer. Thereby, quantification of the isolated RNA was carried out using NanoDrop 1000 spectrophotometer (ThermoFisher, USA) and the quality was assessed by a Qubit fluorometer. Samples with RNA integrity above 7 were assessed using an Agilent bioanalyzer and used for the library preparation. Kapa Hyperprep kit (Roche) was used to produce the cDNA libraries of the six PBMC samples as per the guidelines provided by the manufacturer. The quality of the libraries was checked using Agilent Tapestation. Libraries were pooled and paired-end sequencing on a Novaseq 6000 platform (2X100 bp length) for all of the 6 PBMC samples.

2.4 Data processing and analysis
Clean reads were aligned to the bovine reference genome, ARS-UCD1.2 version 106 (http://ftp.ensembl.org/pub/release-106/gtf/bos_taurus/Bos_taurus.ARS-UCD1.2.106.gtf.gz) and indexed with Bowtie v2.3.5.1 program. The transcript abundance or the expression values was quantified or assembled using RNA-Seq by Expectation Maximization (RSEM) normalized based on Transcripts Per Million (TPM). The read counts or the gene counts of each mRNA generated by the RSEM were used for downstream analysis. After rounding these gene counts values to the nearest integer, the matrix is generated (rows = genes, columns = samples) to identify differentially expressed genes using edgeR. Using the gene count matrix generated in the previous step, differential expression (DE) analysis between control and LPS-challenged was done in the edgeR package. Genes were marked as differentially expressed if the Padj-value was < 0.05, log2 foldchange was either ≤ -1.5 or ≥ 1.5 and the FDR was < 0.05. The relative gene expression of CCT8 & CD36 genes was validated by Real-time PCR (RT-qPCR) analysis. Table 1 contains information on the primers.

Table 1: List of the primers for RT-qPCR validation of RNA-Seq results

Genes	NCBI accession ID	Oligonucleotides (5´-3´)	Amplicon size (bp)	Tm (℃)
CCT8	NM_001033609.1	F- CCTGTCAGTTTCAGAGGTCATAG	115	58
		R- CACTTCATCAACATCCCGTAGA		
CD36	NM_001278621.1	F- CCTTACAATAATACTGCGGATGGA	106	60
		R- AGGAGAGATTCTTTCTGCCTTTG		
GAPDH	NM_001034034.2	F-GTCTTCACTACCATGGAGAAGG	201	60
		R-TCATGGATGACCTTGGCCAG		

Small Ruminant Research 2014; 121(2-3); pp.244-247.

3. Results and Discussion
In this study, we used Peripheral blood mononuclear cells (PBMCs) as pattern recognition receptors and stimulated them with LPS to investigate the global transcriptional response variations between the PBMCs of control versus LPS virulent factor stimulated in Vrindavani cattle by using the high-throughput RNA-Sequencing (RNA-Seq) analysis. Our data support previous studies that have shown the validity of gene expression results acquired utilizing PBMC in various livestock [6, 9]. The RNA-Seq technology combined with

bioinformatics analysis allows us to explore the pathogenic role and pathways of a pathogenic factor more rapidly and comprehensively through probing gene expression profiling [11]. Our study revealed 358 differentially expressed genes (DEGs) as significantly upregulated by meeting the screening. These upregulated genes were subjected to ortholog mapping with Homo sapiens as the target organism using the g: profiler tool. Thereafter, the functional annotation of the enriched gene ontology (GO) pathway: the immune system process, was analyzed using the Cluego tool and represented the network of the attributes through Cytoscape (Fig. 1). The upregulated DE mRNAs potentially modulates the expression of FFAR2, CD36, RAET1G, RAET1E, CEACAM1, SERPINB4, ULBP1, ULBP2, ULBP3, GZMM, CCR2, CCT8, CD200R1, CXCL10, C3AR1, CD200R1, EDNRB, CXCL9, GATA6, GNLY, LYZ, NRG1, PLA2G5, C1QB, C1S, C5AR2, and IGLV3-19 genes. These genes play a vital role in the positive regulation of cytokine production involved in immune response, natural killer cell-mediated immunity, natural killer cell-mediated cytotoxicity, leukocyte mediated cytotoxicity, macrophage migration, T-cell migration, regulation of lymphocyte migration, antimicrobial humoral response, regulation of humoral immune response and regulation of complement activation.

The CD36 and CCT8 genes (selected randomly) were upregulated with log2 fold change (Log$_2$FC= 2.26 and 5.97 respectively). The fold change obtained by the $2^{-\Delta\Delta Ct}$ method of RT-qPCR was compared with the log2 fold change value obtained from the gene expression study carried out through RNA sequencing data analysis. Results revealed in concordance (Fig. 2). The cluster of differentiation 36 (CD36) gene encodes a glycoprotein that acts as a receptor for a broad range of ligands including collagen, low-density lipoprotein, anionic phospholipids, and long-chain fatty acids. CD36 is a member of the cell surface protein class B scavenger receptor family and plays a pivotal role in pathogen recognition, pathogen-induced signaling, and phagocytosis of apoptotic neutrophils. Previous research has revealed that the CD36 mRNA expression considerably rises in cattle during early lactation, indicating the crucial function of this receptor in fatty acids absorption and milk fat synthesis [2]. Through polymorphism mapping, it has been discovered that CD36 and CD36-like genes are involved with the composition of milk fat in goats [16]. Several studies have reported the vital role of CD36 in mediating lipopolysaccharide (LPS)-induced signaling cascades and the internalization of *E. coli* through TLR4 in mammary gland epithelial cells [3]. This is a novel treatment strategy for E. coli mastitis in the early stages of infection. Whereas the Chaperonin TCP1 Subunit 8 (CCT8) gene modulates the theta subunit of the CCT chaperonin, which aids in protein folding in the eukaryotic cytosol. It is also responsible for the transportation and assembly of newly synthesized proteins. Single nucleotide polymorphisms (SNPs) in the CCT8 gene were associated with fertility and production traits in Holstein cattle [1, 4]. Recently, this gene has also been found to be correlated with somatic cell score (SCS) in water buffalo [8]. Somatic cell score (SCS) is an important indicator of resistance and susceptibility of cows to mastitis and can be used to monitor the level of occurrence of dairy cow mastitis.
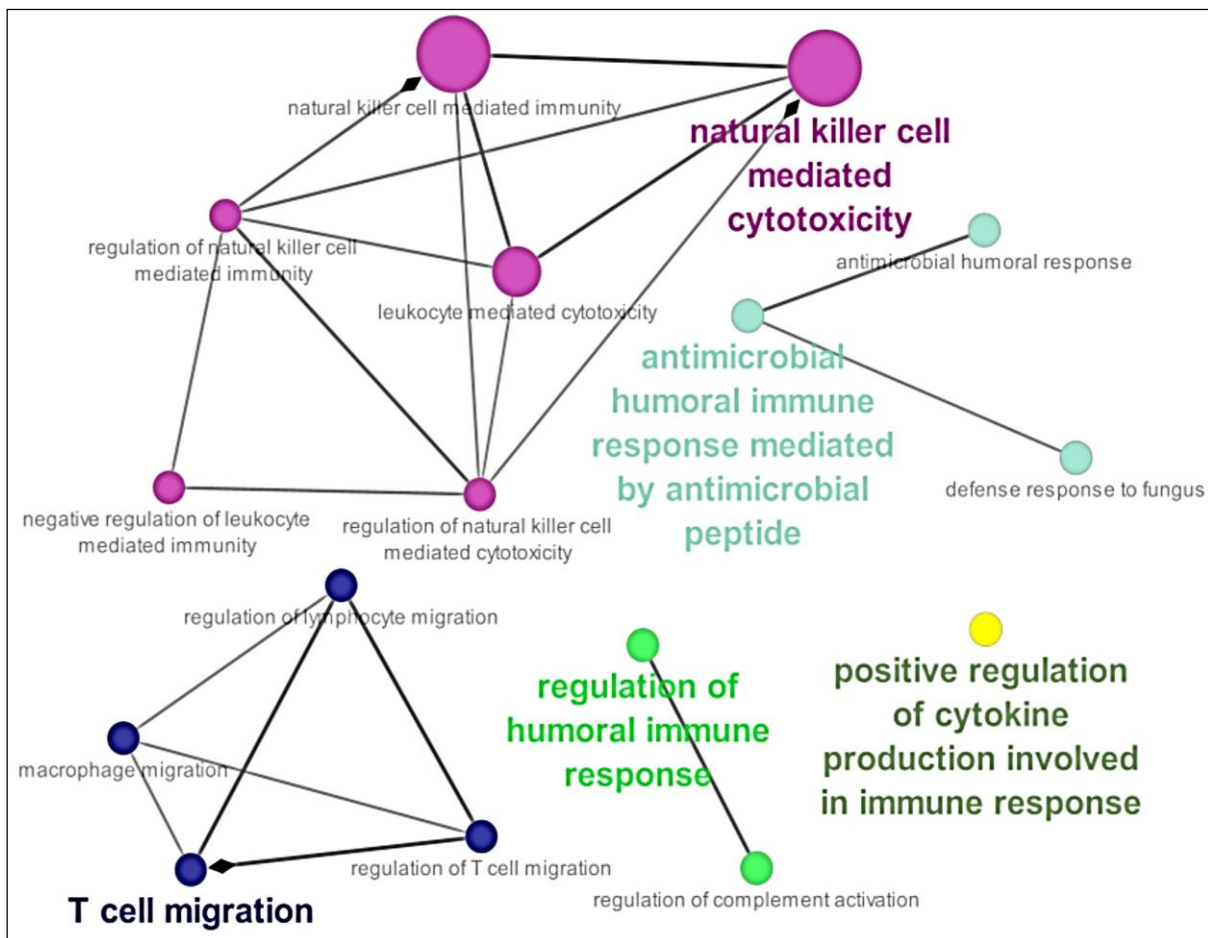

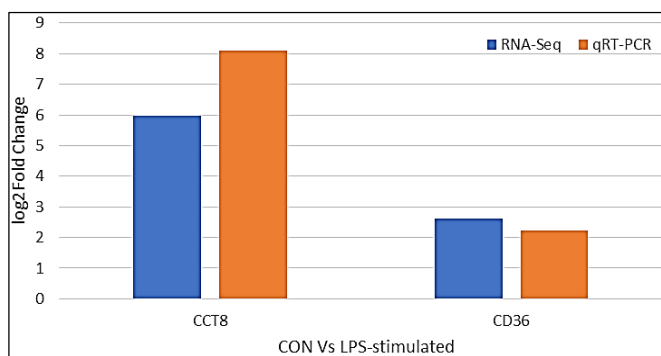**Fig 1:** Pathways significantly upregulated after LPS stimulation

Fig 2: Validation of differentially expressed genes (*CCT8* and *CD36*) by RT-qPCR

4. Conclusion

Keeping in view the fact that *Escherichia coli* is a well-recognized major pathogenic gram-negative bacterium causing bovine mastitis, LPS a key virulence factor of *E. coli* is important in inducing mastitis. We, therefore, need to understand thoroughly the factors influencing the interaction among the etiological agents and the mammary gland cells. As a result, we must identify those differentially expressed genes in response to a specific condition. Modulation of the host-gene expression in response to LPS stimulation was evident from a substantial number of differentially expressed host genes between the control versus LPS-induced experimental group. Narrowing down to the immune-related upregulated-gene expression study reveals pathways associated with the positive regulation of cytokine production involved in immune response, natural killer cell-mediated immunity, macrophage migration, T-cell migration, regulation of lymphocyte migration, antimicrobial humoral response, and regulation of humoral immune response. Gene network analysis of these DEGs plays an important role to understand the interaction among genes. By highlighting the genes involved in the immune system process, we can gain a better understanding of the effect of LPS on the immune system response of dairy cattle peripheral blood mononuclear cells. Through understanding molecular pathways and validating specific genes involved with bovine mastitis, our study revealed PBMCs influenced by LPS transcriptome profile changes. These changes may have a substantial impact on the management of inflammation. However, further and detailed studies of key genes playing a role in inflammation, repetition of experiments, and inclusion of more animals are also required to come to a sure conclusion that may uphold the outcome of a susceptible or tolerance/resistant pattern of the host suffering from bovine mastitis.

5. Conflict of interest

The authors declare no conflicts of interest.

6. Acknowledgment

7. References

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