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Analysis of differential expression of microRNA, bta-miR-93 in lipopolysaccharide challenged peripheral blood mononuclear cells of cattle[#]

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Abstract

The present study was undertaken to analyse the differences in the microRNA (miRNA) expression in lipopolysaccharide (LPS) challenged and unchallenged peripheral blood mononuclear cells (PBMCs) of cattle. The research work was carried out in adult apparently healthy female crossbred cattle maintained at the University Livestock Farm and Fodder Research and Development Scheme, Mannuthy. The expression of miRNA and bta-miR-93 was profiled in the present study by qRT-PCR assay. In silico analysis was done using various online bioinformatic tools for the prediction of target genes, gene ontology analysis and also to study the cellular pathways associated to the target genes of bta-miR-93. The expression analysis of bta-miR-93 showed decrease in its expression in LPS treated PBMCs when compared to control cells. Besides, significant enrichment of target genes of bta-miR-93 was noticed in many immune-related GO terms and in critical immune-associated cellular pathways. The findings of the current study will help in understanding the regulatory role of bta-miR-93 in LPS-mediated immune responses in cattle.

Keywords: M*icroRNA, real-time PCR, PBMCs, lipopolysaccharide*

[#]*Part of MVSc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala*

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MicroRNAs (miRNAs, miR) are endogenous, small non-coding RNAs approximately 20-22 nucleotides long which play a key role in the regulation of gene expression by affecting translation and post transcriptional mechanisms (O'Brien *et al*., 2018). They generally operate by binding to messenger RNA's (mRNA) complementary target regions and impeding or changing the translational machinery. This prevents or modifies the synthesis of the associated protein products. In mammals, miRNAs perform a variety of physiological actions that have been conserved across the course of evolution in development like cell signalling, different stages of the cell cycle, immune cell functions and apoptosis (Mehta and Baltimore, 2016).

Numerous arguments suggest that miRNAs play a significant role in both innate and adaptive immunity. The genesis, differentiation, survival, and activation of B and T lymphocytes, dendritic cells, macrophages, and other immune cell types are dependent on a variety of miRNAs (Gracias and Katsikis, 2011). These are a few of the major functions of immune-related miRNAs, according to recent findings. It is now more evident from numerous studies that dysregulated miRNA expression in the immune system may influence various stages of the pathogenesis of cancer and important bacterial and viral diseases (Croce, 2009).

Considering the regulatory role of miRNAs in several immune mechanisms, the present study was envisaged to find out the differential expression of bta-miR-93 in bovine PBMCs in response to bacterial endotoxin, lipopolysaccharide (LPS) challenge.

Materials and methods

Isolation of peripheral blood mononuclear cells (PBMCs)

The current study was conducted in three apparently healthy adult crossbred cows, maintained at University Livestock Farm and Fodder Research and Development Scheme, KVASU, Mannuthy. Isolation of PBMCs was done using the density gradient centrifugation using Hisep™ lymphocyte separation medium, according to the manufacturer's instructions.

Assessment of cell viability

Viability of cells were evaluated using trypan blue dye exclusion assay with 0.4 percent trypan blue dye and PBMC suspension in a 1:1 ratio. The viable cells were counted in a haemocytometer under low power of microscope. The percentage of viable cells was calculated as follows:

$$\text{Viable cells (\%)} = \frac{\text{Total no. of viable cells per ml of aliquot} \times 100}{\text{Total no. of cells per ml of aliquot}}$$

Culturing of PBMCs

The isolated PBMCs were suspended in sterile Roswell Park Memorial Institute (RPMI) 1640 medium with 10 per cent low-endotoxin heat-inactivated foetal calf serum (Himedia), 2 m*M* L-glutamine (Gibco, Thermo Fisher Scientific), 25 m*M* HEPES buffer (Himedia), 1 m*M* sodium pyruvate (Sigma-Aldrich, St. Louis, MO), 50 µg/mL streptomycin and 50 U/mL penicillin to achieve a concentration of 2×10^6 viable cells per each well of the 12 well culture plate. Cells in treatment group were induced with lipopolysaccharide (10 µg/mL). The cells were incubated as triplicate in 5 per cent CO_2 humidified air for 5 hours at 37 ºC. After incubation the collected cells were harvested by centrifugation and the pellet was collected for quantification of the microRNA bta-miR-93.

qRT-PCR assay of bta-miR-93

Real-time PCR assay using SYBR green chemistry was used to measure the expression of bta-miR-93 in the treatment and control groups. Relative quantification was carried out by normalising the expression of bta-miR-93 to that of endogenous miRNA (miR-191) in the LPS treated and untreated PBMC cultures.

Total RNA was isolated from both study groups using Trizol method. By using spectrophotometry (NanoDrop 2000C), the total amount of isolated RNA was quantified, and its purity was assessed. Poly A tailing of the total RNA from the respective study groups was

performed by using *E. coli* poly A polymerase kit (Poly A polymerase (5000 U/mL), 10X reaction buffer and 10 Mm ATP) purchased from New England Biolabs. The reaction was done with approximately one microgram of total RNA extracted from the respective experimental groups, 0.2 µL of poly A polymerase, 1 µL of 10X reaction buffer and 1 µL of 10 mM ATP. Poly A tailing was carried out in the Biorad T100 thermal cycler by incubating the mix at 37 °C for 30 minutes, followed by five minutes of heat inactivation at 65 °C.

Using specific custom designed RT primers (Kang *et al*., 2012) and M-MuLV Reverse Transcriptase (200 U/µL) (Thermo Scientific), the cDNAs of bta-miR-93 and endogenous control bta-miR-191 were synthesised from polyadenylated RNA samples. The details of the custom synthesised RT primers used in the study are presented in Table 1.

The cDNAs of both the bta-miR-93 and the endogenous miRNA control were synthesised by using 2µL of poly A product, 2µL of RT Primer (10 m*M*/µL), 10 m*M* dNTP and 8 µL of 5X reaction buffer along with 0.5 µL M-MuLV reverse transcriptase by incubation at 42 °C for one hour followed by heat inactivation at 85 °C for 5 min.

Relative quantification of bta-miR-93 by real time PCR

Using Biorad Sso Advanced Universal SYBR green supermix in the Biorad CFX Opus 96 real-time PCR system, the relative quantification of the expression of bta-miR-93 was performed. Custom-designed specific forward and universal reverse primers were used in the qPCR assay of bta-miR-93 according to Kang *et al*. (2012). Table 2 depicts the details of the qPCR primers for the relative quantification of the bta-miR-93 expression.

Standardisation of qPCR conditions using gradient real time PCR

Gradient real time PCR was performed to find the ideal annealing temperature for the primers to amplify bta-miR-93. For subsequent research, the temperature at which the maximum amplification attained was recorded and qPCR assay was carried out with one microlitre of cDNA mixed with 5 µL of SYBR green master mix and 0.5 µL each of forward and reverse primers. The annealing temperature for qRT-PCR programme was fixed at 60˚C for bta-miR-93 and 62.9°C for endogenous control, bta-miR-191.

The qPCR thermocycler programme included initial denaturation at 95 °C for 10 min followed by annealing at 62.9 °C to 54.9 °C for one minute extension at 65 °C for 5 seconds and final extension at 95 °C for 50 seconds.

Relative quantification of expression of bta-miR-93

Relative quantification of bta-miR-93 was carried out by C_t (Cycle Threshold) comparative method (Livak and Schmittgen, 2001), to estimate and calibrate the mean Ct values of the target miRNA and the endogenous control miRNA from the treatment and control groups for each sample. Results were expressed as Mean ± SE and the ΔC_t

Table 1. Sequences of primers used for cDNA synthesis

Sl. No.	miRNA	Primer sequences
1	bta-miR-93	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTTACCTG
2	bta-miR-191	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTCAGCTG

Table 2. qPCR primers for the relative quantification of the bta-miR-93

Sl.No	Micro-RNA	qPCR primers	
		Forward primer	Universal reverse primer
1	bta-miR-93	CAAAGTGCTGTTCGTG	CAGTGCAGGGTCCGAGGT
2	bta-miR-191	CAACGGAATCCCAAAAG	

values were subjected to t test to evaluate the statistical differences between test and control groups.

In-silico analysis of the miRNA bta-miR-93

Multiple online bioinformatic tools were used for target prediction, gene ontology analysis, and exploration of the pathways related to the predicted targets of bta-miR-93.

Target prediction of bta-miR-93

Prediction of targets of bta-miR-93 was done by employing online target prediction tool, TargetScan Human 8.0 (https://www.targetscan.org/). Using the web programme Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/) redundant genes from the list of bta-miR-93 target genes were excluded.

Gene ontology and pathway analysis of the targets of bta-miR-93

Gene ontology analysis and studies of pathways associated with targets of bta-miR-93 in LPS challenged and unchallenged PBMCs were performed by DAVID bioinformatics web tool (https://david.ncifcrf.gov/). By gene ontology analysis, the predicted targets of bta-miR-93 were annotated to biological processes, cellular component, and molecular function categories. Pathway analysis programme (KEGG) of DAVID database was used to analyse and interpret the pathways connected to the predicted targets of bta-miR-93.

Results and discussion

Cell culturing

Peripheral blood mononuclear cells isolated from the blood samples of cattle by density gradient centrifugation showed a viability of more than 90 per cent in the current study. More than 90 per cent viability was observed for all the samples by trypan blue dye exclusion assay.

The collected PBMCs were subjected for cell culturing. Navas *et al*. (2019) recommended more than 90 per cent viability for immunological research using LPS challenged human PBMCs.

Quantitative real time PCR analysis of bta-miR-93

The real time quantification of the expression of bta-miR-93 of the present study revealed decrease in its expression in PBMCs treated with LPS when compared to untreated samples. The fold change observed was 0.318 and was found to be statistically significant ($P<0.05$). Amplification plots and melt curve analysis of bta-miR-93 are depicted in Fig.1 and Fig.2, respectively. Fig.3 depicts the fold change of relative expression of bta-miR-93 in LPS treated and control groups. In contrast to our study, substantial upregulation of miR-93 was reported in nasopharyngeal carcinoma patients (Sun *et al*., 2020) and in canine osteosarcoma (Gourbault and Llobat, 2020).

In-silico analysis

Target prediction of bta-miR-93 performed using the online programme Targetscan human 8.0, revealed 1297 target genes. Singh *et al.* (2016) and Choi *et al.* (2021) had also used TargetScan for the prediction of targets of microRNAs. On GO analysis, these target genes were annotated into different biological processes, cellular component, and molecular function categories. Important GO terms associated with targets of bta-miR-93 in the biological process category were Fc receptor mediated stimulatory signalling, regulation of transforming growth factor beta activation, interleukin-1-mediated signalling and negative regulation of hippo signalling. In the cellular component category, important GO terms associated with targets of bta-miR-93 were SMAD protein complex, astral microtubule, protein phosphatase type 1 complex and phagophore assembly site membrane. Likewise, in molecular function category, methyl cytosine dioxygenase activity, platelet-derived growth factor receptor binding, 1-phosphatidylinositol binding and JUN kinase kinase activity were significantly enriched with targets of bta-miR-93. The top ten significantly enriched immune related GO terms in the biological process category are depicted in Fig.4. Jaeger *et al*. (2017) undertook gene ontology analysis of differentially expressed miRNAs in swine mammary epithelial cells and the results were in agreement with those

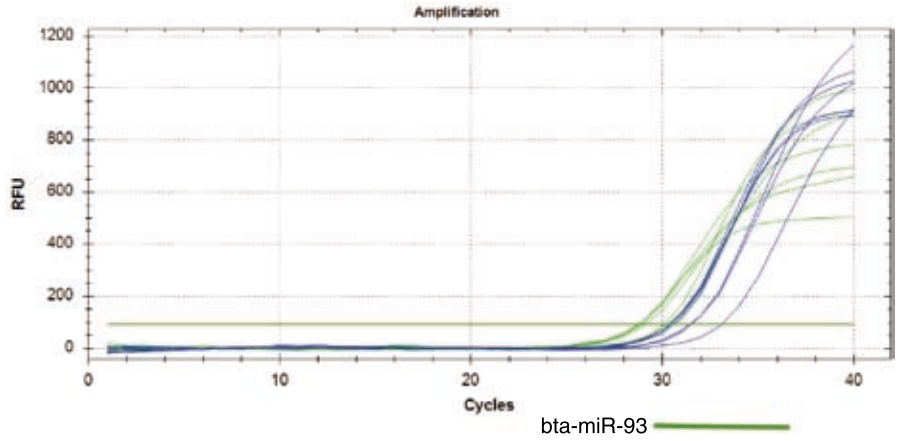

Fig. 1. Amplification plot for the relative expression of bta-miR-93 and the endogenous control bta-miR-191

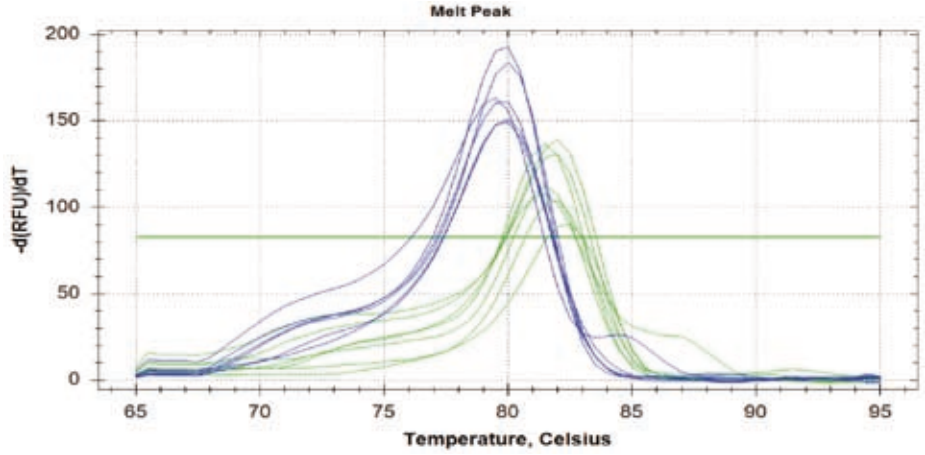

Fig. 2. Melt curve analysis for the relative expression of bta-miR-93 and the endogenous control bta-miR-191

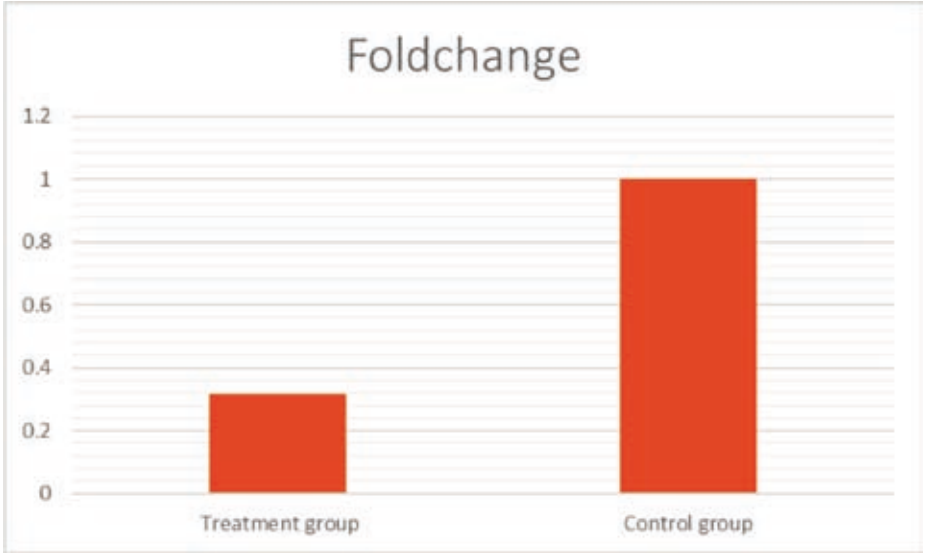

Fig. 3. Fold change for the relative expression of bta-miR-93 in LPS treated and control groups

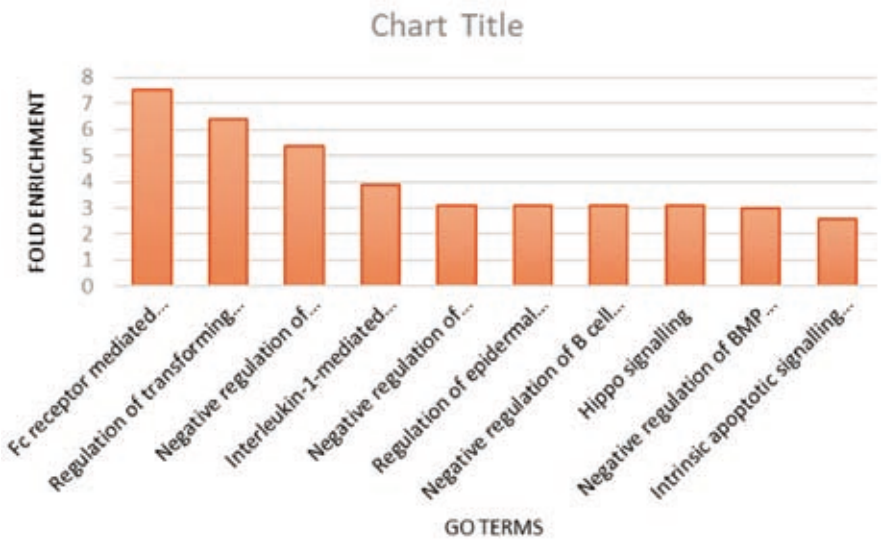

Fig. 4. Top ten significantly enriched immune related GO terms in biological processes category in bovine PBMCs in response to LPS challenge

of our research. According to Mach *et al*. (2016), who used enrichment analysis to find "biological process" gene ontology (GO) terms, about 1,165 differentially expressed genes over-expressed after exercise in horses were strongly linked to the inflammatory response, intestinal permeability, and regulation of the response to stress and bacteria.

Pathway analysis of targets associated with bta-miR-93

According to KEGG pathway analysis, targets of bta-miR-93 were enriched in several immune related cellular pathways with default DAVID parameters. The top ten functionally enriched immune related pathways of targets of bta-miR-93 are presented in Fig.5. The

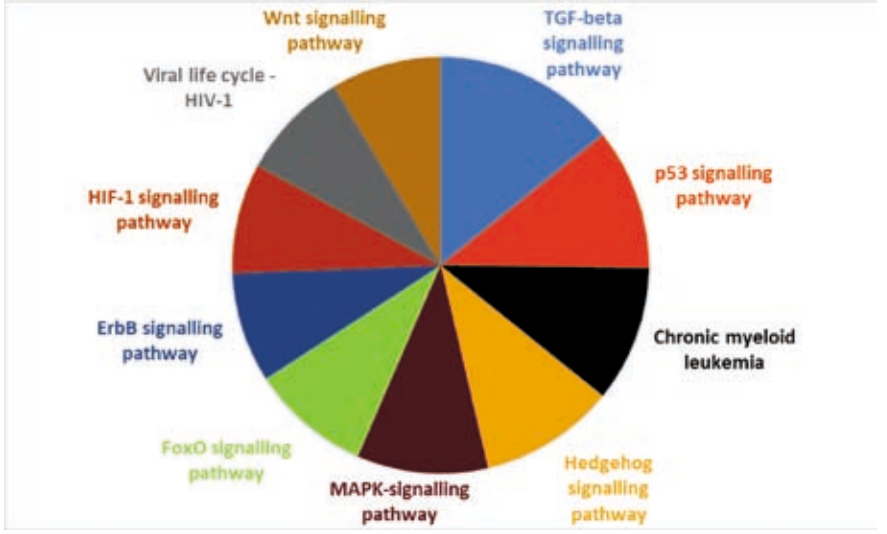

Fig. 5. The top ten functionally enriched immune related pathways of targets of bta-miR-93 in bovine PBMCs in response to LPS challenge

immune signalling pathways especially, TGF-beta signalling, p53 signalling, chronic myeloid leukemia, Hedgehog signalling and MAPK-signalling were significantly enriched with the targets of bta-miR-93. Parkinson *et al*. (2016) and Zhang *et al*. (2020) also identified significant enrichment of targets of differentially expressed miRNAs in LPS treated equine and porcine PBMCs, respectively in many immune mechanisms related to the pathogenesis of diseases.

The top enriched GO terms and pathways associated with the bta-miR-93 targets of the present study are either directly or indirectly connected to many critical immune related mechanisms. LPS treated and untreated bovine PBMCs exhibited significant differences in these mechanisms suggestive of importance of bta-miR-93 in regulation of immune responses

.**Conclusion**

Outcomes of the present study revealed differences in the expression of bta-miR-93 in bovine PBMCs in response to LPS challenge when compared to the untreated control groups. Gene ontology analysis and studies on cellular pathways involved also supported the role of miRNA in regulation of several immune associated mechanisms. The findings of the present research work will shed light for future microRNA based studies on regulation of immune mechanisms in cattle.

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Conflict of interest

The authors declare that they have no conflict of interest.

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