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Analysis of Differential Expression of microRNA, bta-miR-451 in Lipopolysaccharide Challenged Peripheral Blood Mononuclear Cells of Crossbred and Vechur Cattle

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ABSTRACT

MicroRNAs (miRNAs) are endogenous small non-coding RNA molecules, which participate in major cellular processes including metabolism, cell signalling and mechanisms associated with immunity. They have been reported to play an immense role in the regulation of gene expression through their effect on translational repression and gene silencing mechanisms. Recent studies reported the involvement of miRNA, miR-451 in various immune related and cell signalling pathways in many species. As an attempt to understand the regulatory role of miR-451 in immune responses, the present study was undertaken to analyse the differential expression of bta-miR-451 in the peripheral blood mononuclear cells (PBMCs) of crossbred and Vechur cattle after challenging the cells with bacterial endotoxin; llipopolysaccharide (LPS) which acts as the strong stimulators of innate immunity. Significant up regulation was noticed in the expression of bta-miR-451 in LPS stimulated PBMCs of Vechur cattle compared to that of crossbred cattle. Pathway analysis as well as protein- protein interaction (PPI) network analysis of the predicted targets of bta-miRNA-451 also revealed significant association of the miRNA on various immune related mechanisms. As native cattle breeds are supposed to be having high disease resistance, heat tolerance and general adaptability compared to the existing crossbred cattle population, the findings of the present study may contribute in understanding the regulatory role of miRNAs in conferring immunological sturdiness to Vechur cattle, one of the indigenous cattle breed of Kerala.

HIGHLIGHTS

- Significant differences in the expression of miRNA, bta-miR-451 between LPS treated PBMCs of crossbred and Vechur cattle
- Association of target genes of bta-miR-451 in immune related pathways
- Support alleged disease resistance potential of native cattle breeds

Keywords: microRNAs, lipopolysaccharide, Vechur cattle, peripheral blood mononuclear cells, differential expression

MicroRNAs are short, non-coding RNAs, which post-transcriptionally regulate gene expression and are supposed to play a key role in the regulation of innate and adaptive immunity. Studies have reported that miRNAs significantly influence molecular signalling pathways including development, differentiation and functioning of immune cells (Lawless *et al.,* 2014). The mechanism of action of these immune related miRNAs was found to be associated with its role in regulating the expression

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of multiple immune related genes. Recent research findings also revealed that miRNAs play a key role in the pathogenesis, as well as progression of many bacterial as well as viral diseases and also serve as biomarkers of various diseases (Do *et al*., 2021). Among the widely studied group of immune related miRNAs, miR-451 was reported to be having crucial role in regulation of immune responses and associated cell signalling pathways (Bandres *et al.,* 2009; Rosenberger *et al.*, 2012).

Considering the significant role of miR-451 in mediating immune responses, the present study was designed to analyse the differential expression of bta-miR-451 in PBMCs of crossbred and Vechur cattle in response to bacterial endotoxin LPS. Quantitative real time PCR assay followed by *in-silico* analysis for the identification of target genes as well as functional annotation, pathway analysis and PPI network analysis of the predicted targets of bta-miR-451 were carried out. Significant up regulation was noticed in the expression of bta-MIR-451 in Vechur PBMCs compared to that of crossbred cattle in response to LPS challenge. *In-silico* analysis of predicted targets also revealed significant enrichment of targets of bta-miR-451 in many immune related and cell signalling pathways. The differences noticed in the expression of bta-miR-451 between the LPS stimulated PBMCs of crossbred and Vechur cattle is expected to shed light in understanding the regulatory role of miRNAs in conferring the alleged disease resistance in Vechur cattle compared to crossbred animals.

MATERIALS AND METHODS

Isolation, Culture and Stimulation of PBMCs

Peripheral blood mononuclear cells were isolated from the blood samples of crossbred and Vechur cattle by density gradient centrifugation using Hisep 1077 (Himedia, Mumbai, India). The harvested cell populations were evaluated by differential cell count to ensure the predominance of lymphocytes. Cell viability was assessed by Trypan blue size exclusion and samples having a viability of ≥ 95 per cent were selected for the subsequent steps. Isolated PBMCs from respective groups of animals were suspended in RPMI 1640 medium with 25 m*M* HEPES buffer (Himedia), 10 per cent low-endotoxin heat-inactivated foetal calf serum (Himedia), 2 m*M* L-glutamine (Gibco, Thermo Fisher Scientific), 1 m*M* sodium pyruvate (Sigma-Aldrich, St. Louis, MO), 50 u/mL penicillin and 50 µg/mL streptomycin to a concentration of 2 x 10^6 viable cells per mL. The PBMCs from crossbred and Vechur cattle were treated with LPS from *Escherichia coli* O111:B4 (Sigma Aldrich) at a concentration of 10µg/mL. This was followed by incubation of the cultures for six hours in 24 well culture plates at 37°C in a humidified incubator with five per cent CO_2. The cells were then harvested by centrifugation and stored in RNA later at 2-8°C for subsequent qRT-PCR experiments.

Quantitative Real Time PCR Analysis

Polyadenylation reaction

Total RNA was isolated from LPS challenged Vechur and crossbred PBMCs using Trizol method and purity of the isolated RNA was checked by spectrophotometric assessment (Nanodrop 2000C; Thermo Scientific). For quantifying miRNA expression, cDNA of bta-miR-451 and endogenous miRNA control (bta-miR-191) was prepared according to Kang method (Kang *et al.,* 2012) involving polyadenylation of the isolated RNA followed by reverse transcription using specific custom synthesized RT primers. Polyadenylation reaction was performed in Biorad T100 thermal cycler using *E. coli* poly A polymerase kit procured from New England Biolabs. The composition of the reaction mix for polyadenylation included approximately one microgram of total RNA extracted from the respective study groups, 0.4 µL of poly A polymerase (5000 U/mL), 2 µL of 10X reaction buffer and 2 µL of 10Mm ATP for a total reaction volume of 20 µL. The reaction was run by incubating the mix at 37 °C for 30 minutes, followed by heat inactivation at 65 ºC for five minutes.

Synthesis of cDNA

Polyadenylated RNA samples from respective study groups were used for first strand cDNA synthesis of bta-miR-451 and endogenous miRNA control with specific custom designed RT primers and M-MuLV Reverse Transcriptase (200 U/µL) (Thermo Scientific). Reaction mix for synthesis of cDNAs of both bta-miR-451 and endogenous miRNA control included 2µL of poly A

product, 2 µL of RT Primer (10m*M*/µL), 1 µL 10m*M* dNTP and 8 µL of 5X reaction buffer along with 0.5 µL reverse transcriptase for a 20 µL total reaction volume. The reaction mix from respective study groups were incubated at 42°C for one hour followed by heat inactivation at 85°C for 5 min. Details of the custom designed RT primers of both bta-miR-451 including endogenous miRNA control (bta-miR-191) are presented in Table 1.

Quantitative real time PCR analysis was performed using Biorad Sso Advanced Universal SYBR green supermix in Biorad CFX Opus 96 real time PCR system. Appropriate internal controls (bta-miR-191) and non-template controls were included. Melt curve analysis was carried out at the end of each run for checking the specificity of primers. The forward primers and universal reverse primer used for qRT-PCR assay were custom designed (Kang *et al.,* 2012) and synthesised for bta-miR-451 and endogenous control miRNA. Details of primers of bta-miR-451 and endogenous miRNA control for qRT-PCR assay are depicted in Table 2.

Standardisation of qRT-PCR Assay

Gradient real time PCR was employed to identify the ideal annealing temperature for the primers to amplify bta-miR-451 and endogenous miRNA control. The qRT-PCR thermo cycling programme for the amplification of miRNAs consisted of an initial denaturation at 95 ºC for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec and annealing at 54.9 ºC to 62.9 °C for 60 sec. Melt curve analysis was carried out at 65°C to 95°C with 0.5°C increment. The reaction mix for real time PCR have 1 µL of respective cDNAs, 5 µL of SYBR green master mix and 0.5 µL of specific forward and reverse primers. By gradient real time PCR, annealing temperature at which maximum amplification attained for both bta-miR-451 and bta- miR-191 was 60°C and subsequent qRT-PCR assay was carried out with this particular annealing temperature.

Relative quantification of expression of bta-miR-451

Relative quantification of bta-miR-451 in LPS treated PBMCs of crossbred and Vechur cattle was carried out by C_t (Cycle Threshold) comparative method. All the data were analysed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and relative quantification was performed by calibrating ΔCt values of bta-miR-451 from LPS challenged Vechur cattle PBMCs (test) with respect to ΔCt values from LPS challenged crossbred cattle PBMCs (control). All the results were expressed as Mean ± SE and the ΔCt values were subjected to t test to determine significant differences between test and control groups.

***In- silico* analysis**

Targets of bta-miR-451 were predicted using the online target prediction tool viz; miRmap (https://mirmap.ezlab.org/) and the redundant targets were identified and removed from the whole list of targets using the online tool Venny 2.1.0. (https://bioinfogp.cnb.csic.es/tools/venny). Functional annotation and pathway analysis of predicted targets of bta-miR-451 were studied through DAVID (Database for Annotation, Visualization and Integrated Discovery) accessible at https://david.ncifcrf.gov/. The PPI network of predicted target genes of immune related pathways associated to bta-miR-451 was constructed visualised and analysed using the STRING database (https://string-db.org/).

Table 1: Primer sequences for first strand cDNA synthesis of bta-miR-451 and bta-miR-191

Sl. No.	miRNA	Primer sequences
1	bta-miR-451	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTAAACTC
2	bta-miR-191	CAGTGCAGGGTCCGAGGTCAGAGCCACCTGGGCAATTTTTTTTTTTCAGCTG

Table 2: qRT PCR primers for bta-miR-451 and internal control miRNA

Sl. No.	miRNA	Forward primer	Universal reverse primer
1	bta-miR-451	AAACCGTTACCATTACT	CAGTGCAGGGTCCGAGGT
2	bta-miR-191	CAACGGAATCCCAAAAG	

RESULTS AND DISCUSSION

Quantitative Real Time PCR analysis

The present study revealed significant up regulation in the expression of bta-miR-451 (fold change; 10.07 and p value<0.05) in LPS stimulated PBMCs of Vechur cattle, when compared to crossbred cattle. Fig.s 1 and 2 represent the amplification plot and melt curve analysis for the relative expression of bta-miR-451, respectively. Similar findings were observed in the studies of Zhang *et al.* (2020) where the authors reported significant up regulation of miR-451 along with other differentially expressed miRNAs in LPS treated porcine PBMCs. Parkinson *et al.* (2017) and Sharma *et al.* (2020) also carried out differential expression analysis of miRNAs in LPS treated equine and ovine PBMCs, respectively. Both studies reported varying levels of expression of miRNAs in LPS treated PBMCs, but differences in the expression of miR-451 were not identified in both studies.

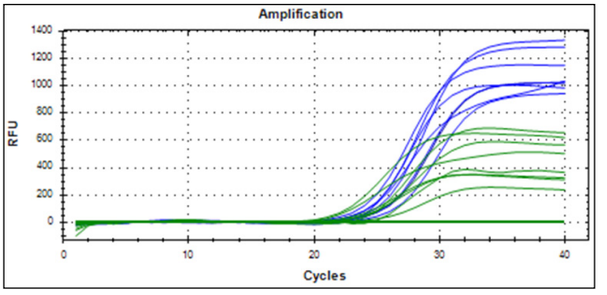


Fig. 1: Amplification plot for the relative expression of bta-miR-451

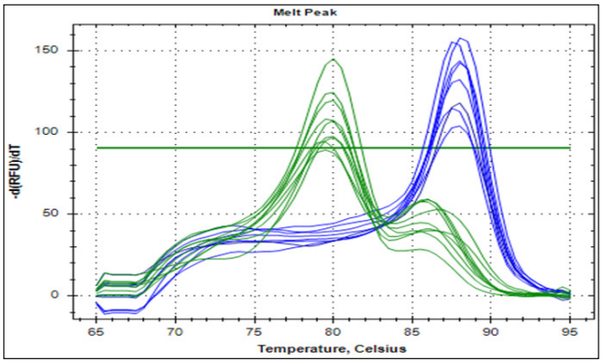


Fig. 2: Melt curve analysis for the relative expression of bta-miR-451

In-silico Analysis

Target prediction

Target prediction of bta-miR-451 by online target prediction tool *viz.* miRmap retrieved 366 targets for bta-miR-451. No redundant targets were identified in the list of predicted targets of bta- miR-451 on analysing the targets using the online tool Venny 2.1.0.

Gene ontology analysis

The gene ontology analysis of the targets of bta-miR-451 through DAVID database annotated 291 targets (87.4 per cent of total targets) in biological process category, 286 targets (85.9 per cent of total targets) in cellular component category and 225 genes comprising of 67.6 per cent of total targets in molecular function category. In the biological process category, significant enrichment of targets of bta-miR-451 was noticed in GO terms associated to major cell signalling and immune related pathways. Highly enriched GO terms in cell signalling pathways were regulation of peroxisome proliferator activated receptor signalling pathway, positive regulation of insulin receptor signalling pathway, negative regulation of transforming growth factor beta receptor signalling pathway, positive regulation of Wnt signalling pathway etc. The significantly enriched immune related GO terms annotated for the targets of bta-miR-451 are presented in Fig. 3.

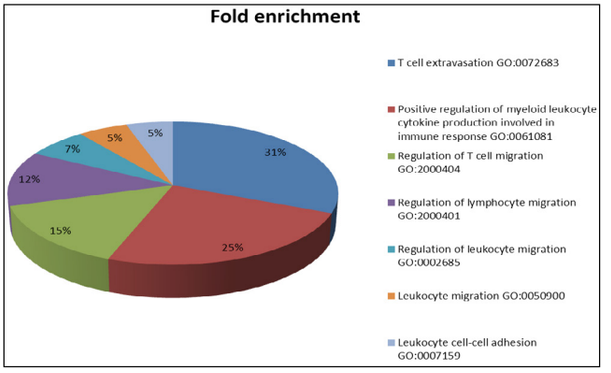


Fig. 3: Significantly enriched immune related GO terms associated to targets of bta-miR-451

Several studies also reported the association of targets of differentially expressed miRNAs from LPS treated

PBMCs in many relevant immune related cellular processes (Parkinson *et al.*, 2017; Zhang *et al.*, 2020 and Sharma *et al.*, 2020). Targets of bta-miR-451 were also found to be associated to many cellular components where, actin cytoskeleton reorganization, nuclear transcriptional repressor complex and H4 histone acetyl transferase complex were noticed to be greatly enriched GO terms associated with the targets of this particular miRNA. According to the studies of Eswarappa *et al.* (2008), LPS was found to be having significant role in reorganization of the actin cytoskeleton in a variety of cells including macrophages and many cell signaling pathways including the NF-κB pathway were influenced by the actin cytoskeleton dynamics. In the present study, significant enrichment of the predicted targets was observed in actin cytoskeleton reorganization which may in turn influence the major cell signalling pathways like NF-κB pathway, one of the important signalling pathways in LPS induced TLR4 signalling (Bhattacharyya *et al.*, 2008).

In the molecular function category, the highly enriched GO terms noticed for the targets of bta-miR-451 were actin filament binding, mRNA binding, double-stranded DNA binding etc. Studies reported that miRNAs interact with the 3′ un translated region (3′ UTR) of target mRNAs to induce mRNA degradation and translational repression (Jacob *et al.,* 2018). However, studies also reported that, miRNAs can also activate translation or regulate transcription where many factors such as subcellular location of miRNAs, the abundance of miRNAs and target mRNAs, and the affinity of miRNA-mRNA interactions affect the basic mechanism (Xu *et al*., 2014).

Pathway analysis

Pathway analysis of predicted targets of bta-miR-451 using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis program of DAVID database also revealed significant enrichment of targets in immune related as well as cell signalling pathways (Fig. 4).

In the present study bacterial invasion of epithelial cells and p53 signalling pathways were found to be most significantly enriched pathways associated to bta-miR-451 targets. The association of bta-miR-451 on development and progression of different types of cancers were reported by Li *et al*. (2013) and Li *et al*. (2018) and the authors reported that up regulation of miR-451 is associated with inhibition cancer cell proliferation. A decrease in cyclin D1, CDK4 and phosphorylated pRB was observed in miR-451 transfected mice tumour cells (Li *et al*., 2018) which can be attributable to the growth arrest in tumour cells but significant changes were not noticed in the expression of the tumour suppressor protein, p53. Li *et al*. (2013) reported that the up regulation of miR-451 is associated with marked down regulation of NF-κB pathway and its downstream targets *viz*; cyclin D1 and c-Myc thus inhibiting tumour progression in human hepatocarcinoma cells. Pathway analysis of the current study also reported significant association of targets of bta-miR-451 in pathways in cancer as well as in p53 signalling.

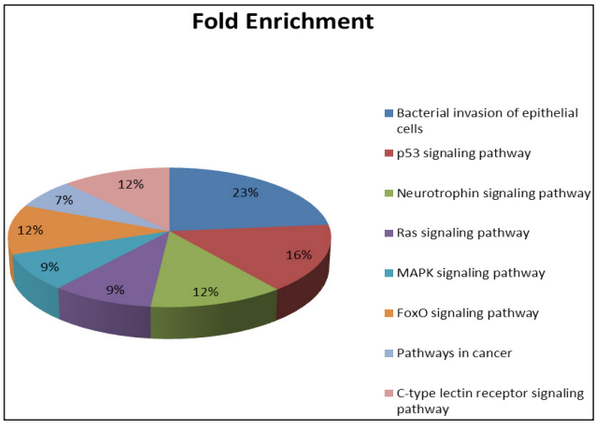


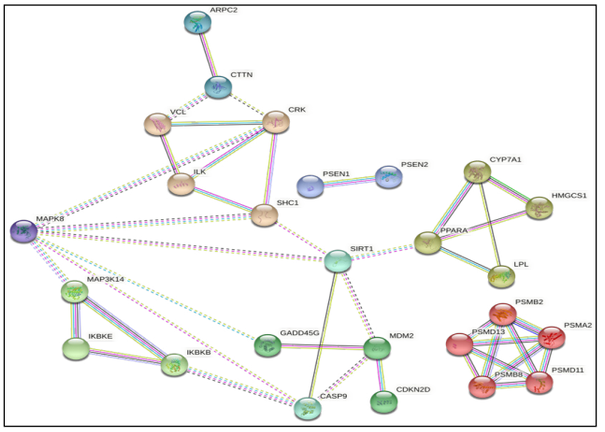
Fig. 4: Significantly enriched pathways associated to targets of bta-miR-451

Many immune related pathways *viz;* pathways in cancer, endocytosis, pathways associated to chemokine signalling, TNF signalling, TLR signalling etc. were reported to be significantly enriched by targets of differentially expressed miRNAs including miR-451 in LPS challenged porcine PBMCs (Zhang *et al.*, 2020). Studies of Naqvi *et al.* (2016) and Sharma *et al*. (2020) also reported similar findings in *in vitro* LPS challenging studies in human and ovine PBMCs, respectively, but differences in the expression of miR-451 was not observed in both studies.

Protein-Protein Interaction Network Analysis

Analysis of PPI networks of immune related targets of bta-miR-451 through STRING database revealed a total of 9 clusters of protein interactions (Fig. 5) and these protein clusters were also found to be having influence

on regulation of immune mechanisms such as, bacterial invasion of epithelial cells, ErbB signalling pathway, neurotrophin signalling pathway, chemokine signalling pathway and regulation of actin cytoskeleton.



Number of nodes: 26; Number of edges: 41; Number of clusters: 9; Average node degree: 2.73; Avg. local clustering coefficient: 0.574; PPI enrichment p-value: 2.04e-09

Nodes represent proteins in the PPI network; Nodes belonging to same cluster represented by single colour; Edges represent protein-protein associations; Solid edges indicates protein-protein associations within a cluster; Dotted edges indicates protein-protein associations between the clusters

Fig. 5: PPI network analysis of immune related targets of bta-miR-451

Functional annotation, pathway analysis as well as PPI network analysis of predicted targets of bta-miR-451 revealed significant role of this miRNA in major cell signalling and immune related mechanisms. These findings were also supported by the studies of miR-451 in human population. Bandres *et al.* (2009) reported that miR-451 regulates the production of macrophage migration inhibitory factor and reduces cell proliferation and increased sensitivity to radiotherapy in human gastrointestinal cancer cells and the authors also reported that down-regulation of miR-451 was associated with worse prognosis. The immunity associated roles of miR-451 were also reported by Rosenberger *et al.* (2012) on pro-inflammatory cytokine responses in mice dendritic cells. The authors reported that down regulation miR-451 expression leads to decreased expression of transcription factors like FOXO3 and ZFP36 resulting in elevated secretion of pro inflammatory cytokines like IL-6 and TNF α.

CONCLUSION

MicroRNAs have emerged as crucial regulators of various biological processes including proliferation and differentiation of cells, cellular signalling mechanisms as well as innate and adaptive immune responses. The present study revealed significant differences in the expression of bta-miR-451 in LPS treated PBMCs of Vechur cattle compared to that of crossbred cattle. Besides, association of predicted targets of bta-miR-451 in various immune related pathways suggest that the miRNA plays a pivotal role in regulation of immune responses among crossbred and native cattle breeds. Findings of the present research work will serve as a basis for future functional studies as well as challenge experiments to unravel the contribution of miRNAs towards the differences noticed in immune responses among the two genetic groups.

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