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Genomic exploration of foot-and-mouth disease signal molecules in Malnad Gidda and Hallikar breeds of Karnataka: A RNA-seq approach

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ABSTRACT

Foot-and-mouth disease (FMD) prevails in India, with a notable increase in incidence in Karnataka state. This infectious malady affects various animals characterized by cloven hooves, including cattle breeds crucial to the rural economy of Karnataka, such as Malnad Gidda and Hallikar. The infection of these breeds with the foot-and-mouth disease virus (FMDV) leads to substantial financial losses for the local population. While previous studies have explored these breeds in combination with foreign counterparts, this research emphasizes a separate examination of Malnad Gidda and Hallikar. This study utilized RNA-Seq data and gene expression analysis, and unveiled a total of 588 differentially expressed genes (DEGs) in FMD-infected Malnad Gidda and Hallikar breeds. Among these, 348 genes were overexpressed, while 240 were under-expressed. The DEGs underwent extensive biological, functional enrichment, and pathway analyses using the DAVID tool. The most enriched terms included 'Defense response to the virus' (GO:0051607), 'Identical protein binding' (GO:0042802), and 'Pathways of neurodegeneration - multiple diseases.' In a network-based analysis, *ATP5PO, GAPDH, ISG15, MX2*, and *PSMD14* were identified as the top hub genes among the significant genes. The study uncovered noteworthy findings indicating that the antiviral capabilities of *ISG15* and *MX2* have been demonstrated in their role against FMDV in both pigs and mice. By delving into the examination of the anti-viral properties of *ATP5PO, GAPDH*, and *PSMD14,* the research establishes a foundational platform for future investigations into FMD, offering potential avenues for interventions in the ongoing quest for effective counter measures against the infirmity in Malnad Gidda and Hallikar Breeds.

Keywords: Expression profiling, Foot and mouth disease (FMD), Hallikar breeds, Malnad Gidda, RNA-Seq

Foot and mouth disease (FMD) is prevalent in India, and higher disease incidents have been perceived in the state of Karnataka (Subramaniam *et al*. 2022). According to the 20th Livestock Census (2019), the state of Karnataka has 8.47 million cattle, 2.98 million buffaloes, 6.17 million goats, 11.05 million sheep, and 0.32 million pigs. This makes the state a major contributor to the total population of animals that are prone to FMD. The Foot and mouth disease virus (FMDV) serotype O evolves at a very fast rate in India, however, Karnataka reported 13 FMD outbreaks in 2020, all attributed to serotype O (Naganayak *et al.* 2022).

Malnad Gidda breeds, known for their resilience and adaptability to challenging weather conditions, are typically reared under a low-input, low-output system, primarily sustaining themselves through grazing (Lohith *et al*. 2020). Similarly, Hallikar breeds are characterized by their medium size, solid musculature, distinctive physical features, and are reared under similar low-cost management practices (Saravanan *et al*. 2021, Singh *et al*. 2008). The studies conducted in Bangladesh found that indigenous breeds of cattle are more prone to FMD than hybrids and exotic animals (Chowdhury *et al.* 2020). However, Indian native breeds such as Malnad Gidda and Hallikar, which play a crucial role in the local rural economy, were reported to be tolerant to many diseases, including FMD (Saravanan *et al.* 2020, Subramaniam *et al*. 2022). Despite their tolerance, any incidences of FMD might impact the rearing of these valuable breeds by farmers, potentially endangering the species.

The urgency to conserve and enhance livestock breeds arises from the threat of FMD, especially to indigenous breeds, necessitating the identification of FMD susceptibility genes. Genetic studies are crucial for understanding FMD pathology, with bioinformatics aiding in identifying key genes, pathways, and regulatory elements (Pereira *et al*. 2020). RNA-Seq offers precise gene

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expression profiling, shedding light on FMD's molecular mechanisms and treatment possibilities (Corchete *et al*. 2020). By analyzing the transcriptomes of Malnad Gidda and Hallikar cattle infected with FMD, this research was carried out to identify molecular markers linked to disease susceptibility and improving targeted interventions.

MATERIALS AND METHODS

RNA-Seq dataset selection: The Gene Expression Omnibus (GEO) database at https://www.ncbi.nlm.nih.gov/geo/ was queried for publicly accessible datasets using the keywords: foot and mouth disease virus, cattle, Expression profiling by high throughput sequencing. RNA-Seq data set GSM5108487, GSM5108488, GSM5108489, GSM5108490 samples of Malnad gidda and GSM5108492, GSM5108493, GSM5108494, GSM5108495 samples of Hallikar was opted for the current analysis, based on their alignment score above 70%. Control samples of Malnad Gidda (GSM5108489 and GSM5108490) and Hallikar (GSM5108494 and GSM5108495) were collected from the ventral soft palate epithelial tissue of *Bos indicus* individuals classified as uninfected.

Comprehensive analysis pipeline: The NCBI Sequence Read Archive (SRA) dataset was accessed (https://www.ncbi.nlm.nih.gov/sra), and SRR records were converted to FASTQ format using sratoolkit.3.0.7 (Sayers *et al.* 2022). Initial quality assessment was done via FASTQC (Brown *et al.* 2017). Trimmomatic 0.39 (Bolger *et al.* 2014) refined data by excluding low-quality reads and adapter contamination. Reads were aligned to ARS-UCD2.0 (*Bos taurus*) using hisat2-2.2.1 (Kim *et al.* 2019). StringTie2 2.2.3 (Kovaka *et al.* 2019) assembled transcripts and quantified expression. Ballgown (Rao 2024) identified Differentially Expressed Genes (DEGs) with a significance threshold of p-value ≤ 0.05 and log2FC ≥ 1.

Strategic GO term analysis: To extract maximum biological significance from the extensive gene lists, DAVID tool was utilized to thoroughly examine them from biological and molecular perspectives (Sherman *et al.* 2022). The limit for determining statistically significant GO terms linked to the DEGs was set at P-values ≤ 0.05.

Building signaling circuitry: The networks are predicted leveraging the STRING database (https://string-db.org/), which employs a relational database system for storing primary data, and final data were filtered with an interaction score above 0.07 (Szklarczyk *et al*. 2023). Utilized the Cytoscape 3.10.2 software, for visualizing molecular interaction network data where centrality analysis was used for the identification of hub genes (Franz *et al*. 2023).

Statistical analysis: R programming version 4.3.2 was employed to perform the statistical analysis. DEGs are identified by utilizing the Bioconductor packages, such as ballgown, devtools, dplyr, and genefilter. For graphical representation, ggplot2 and RcolorBrewer packages from R were employed. The evaluation of gene ontology based on log2-fold change values and P-values was performed using the DAVID tool. Additionally, STRING and Cytoscape 3.10.2 software were utilized for the analysis of protein networks.

RESULTS AND DISCUSSION

The present study intended to identify disparities in gene expression across Malnad Gidda and Hallikar breeds FMDV infected and control samples, as a result, 588 genes were designated as significantly expressed, with a log2-fold change value ≥ 1 and a P-value less than 0.05. Among them, 348 showed up-regulation, while 240 showed down-regulation whereas, individual expression analyses revealed 376 and 50 genes as the most significant in Malnad Gidda and Hallikar, respectively. Fig. 1 depicts the distribution of fold changes between experimental conditions, visually represented by boxplots for differentially expressed genes based on log2fold values. Fig. 2 illustrates the findings of the FMD-related differential gene expression analysis in Malnad Gidda and Hallikar cattle, with an emphasis on the most highly differentially expressed genes. The top

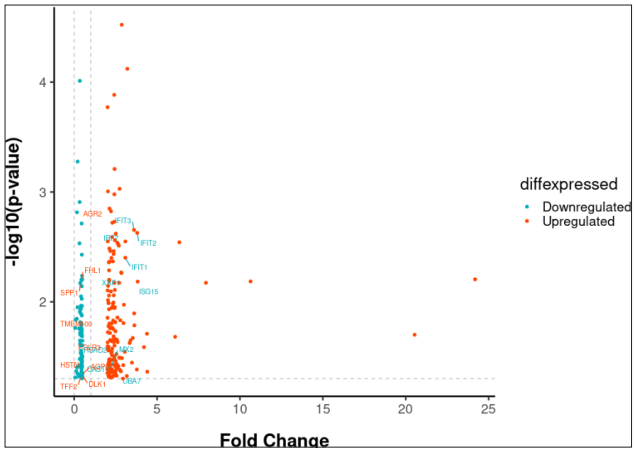

Fig. 1. Box plot illustrating the distribution of log2fold change values for differentially expressed genes of Malnad Gidda and Hallikar experimental conditions (HL, Hallikar; MG, Malnad Gidda; CON, Control and INF, Infected).

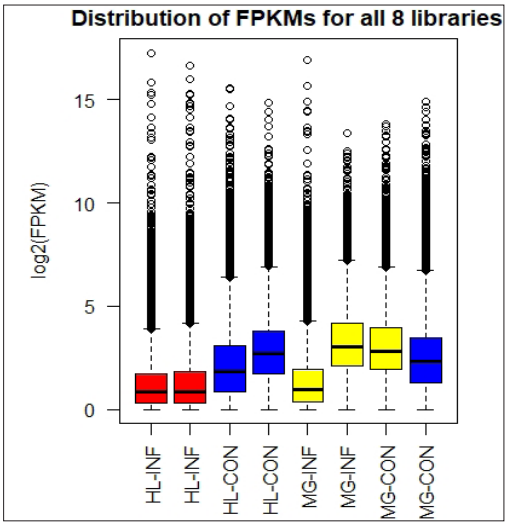

Fig. 2. Plot comparing gene expression in Malnad Gidda and Hallikar cattle regarding FMD. Top genes annotated.

10 significant genes are given in Table 1, among them the top upregulated genes were found to play a crucial roles in FMDV such as, *ISG15*, a cellular protein, inhibits

Table 1. Prime 10 genes showing up regulation and down-regulation

Up-regulated	FDR	Down-regulated	FDR
ISG15	0.00565	*TFF2*	0.00191
MX2	0.00058	*HSTN*	0.04116
IFIT2	0.00985	*AQP9*	0.00167
IFIT3	0.00985	*FHL1*	0.04451
IFIT1	0.00985	*FOLR3*	0.03640
RSAD2	0.00481	*SPP1*	0.04034
OAS1Y	0.00621	*DLK1*	0.01175
UBA7	0.00243	*TMEM100*	0.02323
IRF7	0.00601	*AGR2*	0.02973
XAF1	0.01254	*COL3A1*	0.02631

FMDV proliferation in porcine cells by interacting with the aromatic hydrophobic residue (W105) in the virus's leader proteinase (Lpro), resulting in decreased viral titers (Medina *et al.* 2020). In suckling mice, Hao *et al*. (2021) observed reduced MX2 mRNA expression upon *TPL2* gene deletion, indicating TPL2's influence on antiviral gene expression, which may impact cellular defense against FMD. *IFIT1* belongs to a family of interferon-induced proteins and plays a role in antiviral defense, here the synergistic effect observed in combination with IFN-γ implies that *IFIT1*, contributes to the strengthened antiviral response against FMDV (McDougal *et al.* 2024). In leaderless FMDV infection, *IFIT2* is markedly upregulated (11.5-fold compared to wild-type (WT)) where it binds eIF3, impeding translation and the FMDV Lpro cleaves eIF4G, inhibiting host cap-dependent translation while preserving viral translation via IRES (Kumar *et al.* 2024). The upregulation of *RSAD2* implies its role in antiviral response, highlighting FMDV-host immune system interaction (Yang *et al.* 2022). A significant interferon-stimulated *RSAD2* rise in PBMCs of Malnad Gidda, exhibiting virucidal actions against various viruses, including FMDV (Saravanan *et al.* 2021). At the same time, top down-regulated genes such as, *TFF2* protect the stomach lining, with varying concentrations over a 24 h cycle, potentially impacting ulcer presentation (Banerjee *et al.* 2023), and in bovine, *HSTN* reconfigures gene families linked to milk macromolecular assemblies, affecting milk composition, particularly within the casein gene cluster (Zhou *et al*. 2019). Zhang *et al*. (2021) observed *AQP9* expression in various cow organs, crucial for cattle growth and development. FHL1 found to regulate muscle growth in Qinchuan beef cattle, serving as a marker for selective breeding programs (He *et al*. 2018). García *et al*. (2020) suggested *FOLR3*'s role in facilitating folate uptake from the oviductal environment.

Gene ontology and pathway: GO biological process analysis of significant genes resulted in diverse cellular processes, such as the defense response to viruses including *PYCARD, ZBP1, OAS1Y, IFITM1, ZNFX1, RSAD2, TREX1, MX2, DHX58, IRF7, ADAR, ISG15* (P= 5.69E-05); inhibition of viral genome replication involving *PARP10, OAS1Y, IFITM1, ZNFX1, RSAD2, ISG15* (P= 1.03E-04) ; assembly of mitochondrial respiratory chain complex I comprising *ND6, NDUFB9, NDUFA6, NDUFB3, NDUFB1, ND4* (P= 1.34E-03); translation with *RPS15A, RPS19, RPS29, RPL23, RPL36A, VWA1, RPS27A, RPL39, RPS24, RPS12* (P= 0.0018); ribosomal small subunit biogenesis including *RPS15A, RPS19, RPS27A, RPS24, RPS12* (P= 0.0026); and hydrogen ion transmembrane transport in *ATP5PO, ATP5MC3, SLC25A5, ATP5F1E, ATP5MF, ATP5MC1* (P= 0.0036). Moreover, the identified genes were subjected to GO molecular function analysis, providing insights into

Table 2. Pathways impacted by notable genes in Malnad Gidda and Hallikar

Pathway	Gene
Pathways of Neurodegeneration	*MAP2K3, COX7B, HSPA5, UBA7, NDUFA4, NDUFB3, GPX8, ATP5MC3, COX7A2, NDUFC1, ATP5F1E, TUBA1C, TUBA1A, SLC25A5*
Parkinson's Disease	*COX7B, HSPA5, UBA7, NDUFA4, NDUFB3, ATP5MC3, COX7A2, NDUFC1, ATP5F1E, TUBA1C, TUBA1A, GNAS, SLC25A5*
Coronavirus Disease – COVID-19	*RPL4, RPS26, OAS1X, IFIH1, OAS1Y, RPS29, MX2, MX1, RPL36A, ISG15, RPL37*
Thermogenesis	*MAP2K3, COX7B, NDUFA4, COX17, NDUFB3, GNAS, ATP5MC3, NDUFC1, COX7A2, ATP5F1E*
Diabetic Cardiomyopathy	*COL3A1, COX7B, COL1A2, NDUFA4, NDUFB3, ATP5MC3, NDUFC1, COX7A2, SLC25A5, ATP5F1E*
Chemical Carcinogenesis - Reactive Oxygen Species	*COX7B, GSTM1, NDUFA4, NDUFB3, ATP5MC3, NDUFC1, COX7A2, SLC25A5, ATP5F1E*
Oxidative Phosphorylation	*COX7B, NDUFA4, COX17, NDUFB3, ATP5PO, NDUFC1, COX7A2, ATP5F1E*
Protein Export	*SPCS3, SPCS1, HSPA5, SEC61G, SEC61B, SEC11C*
Glutathione Metabolism	*RRM2, GSTM1, GPX4, ODC1, GAPDH, SRM*
Prion Disease	*TUBA1C, COX7B, TUBA1A, HSPA5, NDUFA4, NDUFB3, ATP5MC3, NDUFC1, COX7A2, SLC25A5, ATP5F1E*

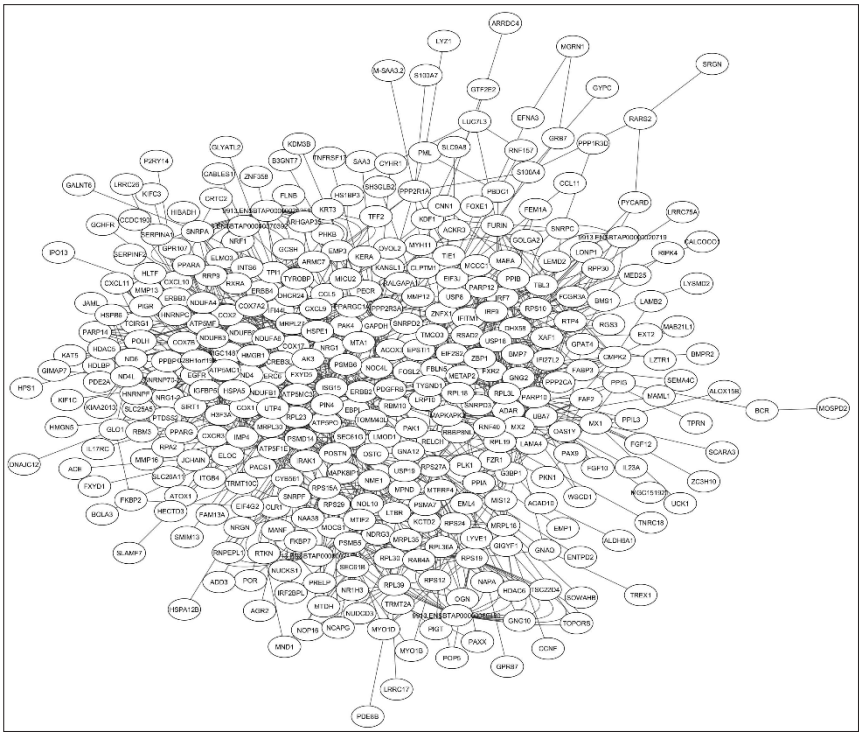

Fig. 3. Network depicting interactions among the most consequential up-regulated DEGs and their associated nodes.

Table 3. List of top 10 genes based on centrality analysis

Betweenness	Score	Degree	Score	Closeness	Score
ATP5PO	0.0411	*ATP5PO*	23	*ATP5MC1*	0.3611
EPSTI1	0.0463	*GAPDH*	53	*ATP5PO*	0.375
ERBB2	0.0595	*ISG15*	58	*ERBB2*	0.3993
GAPDH	0.1924	*MX2*	29	*GAPDH*	0.4074
ISG15	0.1681	*PSMD14*	28	*ISG15*	0.4125
MRPL27	0.0500	*RPS13*	26	*MX2*	0.3907
MX2	0.0752	*RPS19*	29	*NDUFB1*	0.3692
PSMD14	0.0723	*RPS27A*	26	*PPARGC1A*	0.3655
RPS27A	0.0606	*RPS8*	23	*PSMD14*	0.3604
USP18	0.0468	*TPI1*	23	*USP18*	0.363

Table 4. Central hub genes identified through PPI analysis

Gene	Betweenness	Degree	Closeness
ATP5PO	0.041129	23	0.375
GAPDH	0.192479	53	0.407407
ISG15	0.168101	58	0.4125
MX2	0.075212	29	0.390743
PSMD14	0.072392	28	0.360477

their functional roles. Notable categories included double-stranded RNA binding, involving *OAS1X, IFIH1, OAS1Y, DHX58, VIM* (P= 0.0012); GTP binding, encompassing *TUBA1C, TUBA1A, MX2, MX1, GNAS, AK3, GBP4, GEM, PCK2* (P= 0.005); integrin binding, featuring *COL3A1, FN1, SPP1, ISG15, PPIA* (p= 0.0056); structural constituents of the ribosome, comprising *RPL4, RPS26, RPS29, RPL36A, ISG15, RPL37, GAPDH* (P= 0.006968); zinc ion binding, with *IFIH1, MMP12, NR4A3, ZNFX1, ADH1C, RPS29, QPCT, DHX58, ERAP1, ADA, MSRB1, NR3C2* (P= 0.0082); identical protein binding, involving *ADH1C, GPX4, MX1, FN1, DBI, DEFB1, RNASE1, IFIT3, IFIH1, COL1A2, AGR2, GNPNAT1, VIM, RABAC1* (P= 0.0172); and GTPase activity, including *TUBA1C, TUBA1A, MX2, MX1, GNAS, GBP4, GEM* (P= 0.0235). The top biological processes and molecular function of the genes were acquired with a significance level of $P < 0.05$ and detailed results are provided in Supplementary Table 1. Meanwhile, the KEGG pathway analysis employs a stringent P-value cutoff of less than 0.05. The outcome of the pathway analysis unveiled enrichment across various pathways, detailed information about the genes belonging to these pathways can be found in Table 2. These findings underscore substantial enrichment, particularly in pathways associated with various ailments among the DEGs.

Many identified genes are linked to defense against viruses and protein binding. Notably, the study by Zhang *et al.* (2021) and Swaim *et al*. (2020) supports the role of *ISG15* in virus defends, inhibiting viral genome replication,

integrin binding, and contributing to ribosomal structure. *MX2* participates in GTP binding and viral response (Layish *et al.* 2024). Additionally, *GAPDH* aids ribosomal (Shi *et al.* 2023), *PSMD14* regulates ubiquitin-protein transferase activity positively (Bustamante *et al.* 2020), and *ATP5PO* facilitates hydrogen ion transport (Neupane *et al*. 2019). ISG15 and MX2 are involved in the coronavirus pathway, indicating relevance beyond FMDV (Toro *et al*. 2022). *ATP5PO* and *GAPDH* contribute to oxidation phosphorylation and glutathione metabolism pathways, vital for cellular responses to viral-induced oxidative stress.

Hub-gene identification: Protein-protein interaction analysis of the identified DEGs, resulted in a network comprising 1430 nodes and 500 edges as shown in Fig. 3. Topological analysis based on Degree, Betweenness, and Closeness identified *ATP5PO, GAPDH, ISG15, MX2*, and *PSMD14* as the top 5 hub genes based on centrality analysis shown in Tables 3 and 4. Previously discussed *ISG15* and *MX2* functions are substantiated by qRT-PCR studies. Medina *et al.* (2020) and Hao *et al.* (2021) confirmed *ISG15*'s inhibition of Foot-and-Mouth Disease Virus (FMDV) proliferation by interacting with its leader proteinase. *MX2*, known for restricting viral replication, shows reduced expression in TPL2-deleted mice, suggesting a link between *TPL2* regulation and *MX2* during FMDV infection (Medina *et al.* 2020, Hao *et al.* 2021). Furthermore, *ATP5PO, GAPDH*, and *PSMD14* play significant roles in bovine physiology. *ATP5PO* aids ATP synthesis crucial for cellular functions (Zhuang *et al*. 2023). *GAPDH* is essential for DNA tasks, emphasizing genomic integrity (Shi *et al.* 2023). *PSMD14* regulates spermatozoa molecular functions, potentially impacting reproductive processes (Ren *et al.* 2023). These findings underscore the genes' multifaceted importance in bovine physiology, guiding targeted research for understanding FMDV infection mechanisms and developing therapeutics.

The present study emphasizes the potential ramifications of Foot and mouth disease virus (FMDV) on Malnad Gidda and Hallikar breeds in Karnataka, emphasizing *ISG15* and *MX2*, linked to the restraint of FMDV proliferation and additionally, the remaining hub genes, *ATP5PO, GAPDH*, and *PSMD14*, contribute significantly to various physiological processes in bovine organisms. The intricate roles of these genes in ATP synthesis, DNA maintenance, and reproductive pathways underscore their importance in shaping the molecular landscape of bovine physiology. Further exploring the consequences of Foot and mouth disease infections on the antiviral capabilities of these genes is imperative for comprehending the fundamental biology and reproductive health of Malnad Gidda and Hallikar breeds. Overall, it provides a rationale for further research and potential interventions.

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