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Expression profile of CCL2, CXCL10 and IFIT2 genes in FMD vaccinated Tharparkar cattle

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Abstract
Foot-and-Mouth Disease (FMD) poses a significant economic threat globally, with estimates of annual losses ranging from 12,000 to 14,000 crore FMD in India alone. The study evaluates the expression profiles of key immune response genes CCL8, CXCL10, and IFIT2 in Tharparkar cattle post-FMD vaccination. PBMCs samples were collected from the blood at three time points: pre-vaccination (day 0), and post-vaccination on days 7 and 21 for the study and gene expression was analyzed using quantitative real-time polymerase chain reaction (ORT-PCR). Results indicate an upregulation of CCL8 on day 7 and day 21 post-vaccination, aligning with previous findings after FMD vaccine and challenge studies. Similarly, CXCL10 showed an initial upregulation on day 7, however, a downregulation was noted on day 21. IFIT2 exhibited a complex expression pattern, with a decrease on day 7 and on day 21, differing from previous studies. The results provide preliminary insights into the Tharparkar breed's unique response to vaccination, emphasizing the need for further validation with a larger sample size.

Keywords: FMD, PBMCs, ORT-PCR, CCL8, CXCL10 and IFIT2

1. Introduction
Foot-and-Mouth (FMD) disease is highly contagious and important disease with great economic concern causing estimated total annual economic loss ranges from 12,000 to 14,000 crore (Singh *et al.*, 2013) [9] in India. In India at present, inactivated vaccine is used biannually to vaccinate the animals for serotype O, Asia-1 and A. After mass vaccination, still outbreaks occur of FMD in different geographical regions in different frequency (Annual report, 2020-21) [1]. The titer of FMDV-specific neutralising antibodies is commonly used to assess vaccine-induced serotype specific protection (Dus Santos *et al.*, 2000) [5], but low antibody levels can also be protective, and animals with high neutralising titers can develop disease after being challenged (Eschbaumer *et al.*, 2016) [6]. Both the innate and adaptive immune responses are necessary for protection against any pathogen. Before, it was thought to that the innate immune system plays a significant part in the immunological response, while the adaptive immune system is also the factor that influences the immune response (Suresh and Mosser, 2013) [10]. CCL8 and CXCL10 are Chemotactic cytokines (Chemokines) which belong to a broad family of peptides (60-100 amino acids) structurally linked to cytokines and are tiny heparin-binding proteins with the primary function of regulating cell traffic (Deshmane *et al.*, 2009) [4]. Chemokines play a crucial role in the selective recruitment of monocytes, neutrophils, and lymphocytes when they are released in response to signals such proinflammatory cytokines. In peripheral circulation and tissues, these cells are crucial for antiviral immune response (Callewaere *et al.*, 2007) [2]. IFIT2 is an IFN-induced antiviral protein which inhibits expression of viral messenger RNAs lacking 2'-O-methylation of the 5' cap. The ribose 2'-O-methylation would provide a molecular signature to distinguish between self and non-self mRNAs by the host during viral infection (Davis *et al.*, 2017) [3]. Tharparkar is an indigenous dual purpose cattle breed found along with the Indo-Pak border covering Western Rajasthan (Jaiselmer & Barmer) and up to “RUNN OF KUTCH” in Gujrat. This Cattle breed is well known for its disease resistance properties and adaptability to arid climatic conditions of Thar desert. This study was conducted to check expression profile of CCL8, CXCL10 and IFIT2 gene in Tharparkar cattle after FMD vaccination at three different time

point as before vaccination on day 0 and post vaccination on day 7 and on day 21 through ORT-PCR.

2. Materials and Methods

2.1 Blood collection and PBMCs isolation

Initially 10 Tharparkar naïve cattle calves of 4 months of age were selected for blood collection from Cattle and Buffalo breeding farm at IVRI Izatnagar, Bareilly. 5 ml blood was collected in each Heparin coated Vacutainer tube and Normal vacutainer tube for PBMCs collection and serum separation subsequently under sterile conditions before (0 day) vaccination. Calves were vaccinated just after 0 day blood collection with 2 ml intramuscular injection of "RAKSHA-OVAC TRIVALENT" FMD vaccine containing trivalent Foot and Mouth disease inactivated antigens of O, A and Asia-1 strains. DIVA was performed for assurance of absence of any previous FMD infection and SPCE was carried out to check the absence of maternal antibody (MAB) in 0 day serum samples of calves. Calves those were free from any previous FMD infection and maternal antibody, were selected for further blood collection on day 7 and day 21 post vaccination for PBMCs isolation and on day 28 post vaccination to determine protective antibody titer through SPCE in serum samples. After DIVA and SPCE, PBMCs samples were used for the study only of those three Tharparkar calves which were free from any previous FMD infection and MAB at day 0 and had Protective antibody titre in their serum at day 28. PBMCs were isolated by density gradient centrifugation. Selected calves were remained healthy during blood collection for PBMCS and serum without manifestation of any clinical findings for any disease.

2.2 RNA isolation and cDNA synthesis

TRIzol method was used for RNA extraction from preserved 9 PBMCs Samples. Samples were checked for concentrations and quality by taking OD in nanodrop using 1.5 µl of each sample. cDNA was synthesized from RNA extracted in previous step using QuantiTect reverse Transcription Kit. In first step of CDNA synthesis, genomic DNA was whipped out by using DNA wiped out buffer (2 µl), Template RNA (500 ng) and RNase free water. All reaction components were mixed in each PCR tube for final volume of 15 µl and incubated for 2 minutes at 42ºC and then placed on ice immediately. In second step, for cDNA synthesis 20 µl reverse-transcription master mix was prepared for each sample/tube on ice by mixing Quantiscript Reverse Transcriptase (1 µl), Quantiscript RT buffer, 5x (4 µl), RT primer Mix (1 µl) and Template RNA (Entire genomic DNA elimination reaction) (14 µl). Tubes were incubated for 15 minutes at 42 °C and then 3 minutes at 95 °C. Then cDNA samples were preserved at -20 °C until further use.

2.3 Quantification of mRNA by ORT-PCR

Primers (Forward and Reverse) for quantification of genes were designed using NCBI and PrimerQuest tool and checked by OligoAnalyzer tool for size of primers, GC contents, melting temperature (Tm), Secondary structure. Detail of genes and respective primers is given in table 1.

Table 1: Genes and respective primers

Gene name	Forward primer (5' – 3')	Reverse primer (5' – 3')	Amplicon size (bp)
CCL8	GCTCAGCCAGATTCAGTTTCTA	GGTGATTCTCGTGTAGCTGTC	99
CXCL10	AGGTGTACCTCTCTCTAGGAATAC	AGGATTGACTTGCAGGAATGA	110
IFIT2	CAGGGATCAAAGGAAGGAGAAA	TCCTGAAGAAACGCCAAGAG	116
GAPDH	CTGGGCTACACTGAGGACCA	TCCACCACCCTGTTGCTGTA	158

The qPCR reaction was run in Agilent AriaMX real-Time PCR machine with the three step cycling protocol. Thermo Scientific Maxima SYBER Green/ROX qPCR Master Mix (2x) kit was used for qPCR reaction preparation. For quantification of each specific gene, 42 reactions were prepared in each plate (2 technical replicates of 3 control, 3 samples of 7th day post vaccination and 3 samples of 21th day post vaccination for each specific gene and endogenous control with 6 reactions of Non-technical control. The Ct values generated by ORT-PCR machine were used for calculation of log2 fold change expression of genes on 7th day and 21th post vaccination in comparison of 0 day prevaccination/control using GAPDH gene as endogenous control.

3. Results

The Ct values were used to calculate ΔΔCt values and the relative log2 fold change expression. Expression of CCL8 gene was found increased on day 7 and day 21 post-vaccination in comparison of day 0 prevaccination. Expression of CXCl10 gene initially increased on day 7 but on day 21, expression was found decreased in comparison of day 0 revaccination. Expression of IFIT2 gene decreased on day 7 in comparison of day 0 while expression on day 21 was found increased in comparison at day 7 but still was lower than day 0 (Figure 1).

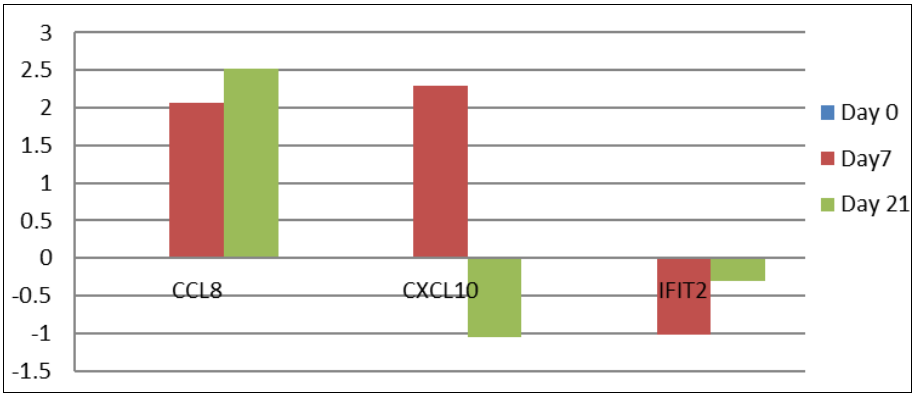

Fig 1: Log2 fold change expression of CCL, CXCL10 and IFIT2 gene on 7 and on day 21 in comparison with day 0.

4. Discussion

CCL8 and CXCL10 are Chemotactic cytokines (Chemokines) which belong to a broad family of peptides (60-100 amino acids) structurally linked to cytokines and are tiny heparin-binding proteins with the primary function of regulating cell traffic (Deshmane *et al.*, 2009) [4]. IFIT2 is an IFN-induced antiviral protein which inhibits expression of viral messenger RNAs lacking 2'-O-methylation of the 5' cap. The ribose 2'-O-methylation would provide a molecular signature to distinguish between self and non-self mRNAs by the host during viral infection (Davis *et al.*, 2017) [3].

In this study we found upregulation of CCL8 on day 7 and day 21 postvaccination. Similar result was found by Zappulla (2014) [12] in his study where he observed upregulation of CCL8 gene after Ad5-FMDV vaccine and FMDV challenge study. Further, upregulation of CXCL10 has been observed in our study observed on day 7 which was similar to study done by Lv *et al.* (2018) [7] in porcine PBMCs collected on day 10 after FMDV infection. Saravanan *et al.* (2021) [8] also observed upregulation of CCL8 and CXCL10 after FMD infection on day 5 in Malnad Gidda and Hallikar cattle. Although down-regulation was observed at day 21 in comparison of day 0 in our study. Expression pattern of IFIT2 gene was not found in accordance with Saravanan *et al.* (2021) [8] where he found upregulation of IFIT2 in his RNA-Seq analysis. This discrepancy may be due differential response of host to vaccination and viral infection as IFIT2 inhibits expression of viral messenger RNAs lacking 2'-O-methylation of the 5' cap and only neutralising antibody (nAb) mediated humoral immunity is activated after vaccination produced by inactivated virus, and nAbs are generated against viral serotypes present in the vaccine (Windsor *et al.*, 2011) [11].

5. Conclusion

In this study PBMCs samples were collected after FMD vaccination while most of previous studies have been carried out after FMD infection in other breeds or species. Expression of CCL8 and CXCl10 gene were in accordance with previous studies except IFIT2. The data provide an initial clue regarding this cytokine's gene expression profile following exposure to FMD vaccination. However, results should be further validated with larger sample size.

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