INTERFERON-γ MESSENGER RNA LEVEL IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF NAIVE BOVINES AFTER FOOT-AND-MOUTH DISEASE VIRUS VACCINATION AND CHALLENGE

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ABSTRACT

The study evaluated the antibody response and interferon-gamma (IFN-γ) messenger ribonucleic acid (mRNA) expression levels in naive bovine calves from a Foot-and-Mouth Disease virus (FMDV) potency test. Peripheral blood mononuclear cells (PBMCs) were separated from blood samples and total RNA was extracted. Two step quantitative (Real time-polymerase chain reaction (RT-PCR) method was used for measurement of IFN-γ specific mRNA. Plasma samples were screened to detect anti-non structural proteins (NSP) antibodies for FMDV in 3AB NSP antibody enzyme linked immunosorbent assay (ELISA). Plasma samples were found free for anti-NSP antibodies for FMDV in vaccinated bovine calves as evaluated in 3AB NSP antibody ELISA test. Increase in mRNA expression level of IFN-γ after 15 days post vaccination (DPV) was found unequivocally in all five vaccinated calves (more than two-fold increase in 4 out of 5 calves). Liquid phase blocking ELISA (LPBE) test results also revealed highest humoral antibody response on 15 DPV. Monovalent FMDV vaccination elicited highest antibody titre on 15 DPV in correlation with elevated IFN-γ mRNA level. Humoral and cellular immunity both play important role in protection against FMDV. The study will help in further understanding the role played by both the humoral and cellular immune response against FMDV.

Keywords: buffalo, *Bubalus bubalis*, blood mononuclear cells, bovine, Foot-and-Mouth Disease, IFN-γ, immunity

INTRODUCTION

Foot-and-Mouth disease is caused by an Aphthovirus classified in the family *Picornaviridae* which has single stranded positive sense RNA as genomic material. Vaccination

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only provides temporary immunity that lasts from 6 to 9 months to years. Efficacy of vaccination is judged predominantly based on serological assays for detecting virus specific neutralizing antibodies by means of the serum neutralization test (SNT). Efficacy of the vaccine, per second, is judged by means of the cattle potency test for 50% protective dose (PD50) of the vaccine as per World Organisation for Animal Health (2009). Vaccination induces both $CD4^+$ and $CD8^+$ T cells. Humoral immunity against FMD has largely been considered the most important factor in protection efficiency, there is no doubt that a vaccine that also stimulates good cellular immunity would enhance this efficiency and perhaps prevent persistence as well as induce a more durable immunity. Recently studies have suggested the role of cell mediated immunity (CMI) response towards FMDV could be used either as a diagnostic test or provide a correlate of protection in animals post vaccination. Specific induction of IFN-γ was reported by Parida *et al.* (2005) in samples from vaccinated, infected and vaccinated-then-infected cattle. The cytokine expression studies, more importantly IFN-γ is vital for understanding disease progression and concurring protective immune response. A better basic understanding of the interactions between FMDV and its host at the level of cellular interactions and immune responses will improve the chances of developing new molecular level identification of the disease progression, new generation vaccines and anti-virals. In this regard cultures of PBMCs were being used to study the cytokine profiles induced by vaccination which can be considered as determinants for the efficacy and characteristics of the developing immune defenses. IFN-γ modulates immune response by inhibiting the viral replication and is produced by T-cells, NK-cells (Bach *et al.*, 1997), B-cells and antigen presenting cells (Frucht *et al.*, 2001; Gessani and Belardelli, 1998). Involvement of IFN-γ mediated pathway is implicated in FMDV infection (Zhang *et al.*, 2002). Investigations in the immune responses elicited by FMDV were needed to improvise the existing vaccines and to sketch novel control strategies against FMD (Summerfield *et al.*, 2009). So, this study was planned with an objective - to analyse and understand the expression of IFN-γ after FMDV infection *vis-à-vis* vaccination.

MATERIALS AND METHODS

Cells and virus

BHK-21 monolayer cells maintained at the Project Directorate on Foot and Mouth Disease (PDFMD) were used for culture of FMDV using MEM (Diffco, USA). The O IND R2/75 vaccine strain maintained as monolayer cultures at PDFMD was obtained and maintained in the virus seed laboratory, Indian Immunological Limited (IIL), Hyderabad as a BHK-21 adapted suspension culture for preparation of antigen used to formulate vaccines with different payloads. Cattle challenge virus O IND R2/75 was prepared from the BHK-21 adapted virus by the FMDV Laboratory, Research and Development Centre, IIL, Hyderabad. These viruses had been passaged and titrated in cattle tongue. The titrated viruses were aliquoted and stored as 50% glycerol stock. For challenge experiments, 10^4 cattle ID_{50} were used for the intra-dermo-lingual challenge of each calf.

Animals

Seventeen Jersey or Friesian crossbred cattle (about 10 months of age) was obtained from the holding farm of IIL, Hyderabad. These animals were reared in the farm from one month of age and

were screened by 3 rounds of testing for FMDV-NSP antibodies using PrioCHECK FMDV NS kit (Prionics Lelystad BV, The Netherlands). All the animals were NSP seronegative in all the three tests. Additionally, the animals were tested for the absence of virus in the oesophago-pharyngeal fluids (probang samples) thrice by virus isolation on primary bovine thyroid cells followed by antigen ELISA (Hamblin *et al*., 1984) and RT-PCR (Reid *et al*., 1999).

Vaccine

Monovalent oil adjuvant vaccine was blended as a single oil emulsion incorporating O IND R2/75 vaccine strain in montanide oil ISA 50V (Seppic, France). The vaccine (2 ml) was administered to cattle calves by intramuscular route.

Potency test

Potency test in cattle calves were carried out as per protocol suggested by the World Organisation for Animal Health (2009) for testing FMD vaccines. In short, three groups of cattle calves were vaccinated with the monovalent oil adjuvant vaccine as neat, 1:5 dilution and 1:25 dilution. Two unvaccinated calves were kept as controls. The animals were challenged on day 21 post vaccination by intradermo-lingual route on two sites using 10^4 cattle ID_{50} O IND R2/75 cattle challenge virus per site. In the present study, only the samples from neat monovalent vaccine administered and control calves were used.

Blood and plasma samples

Blood samples were collected from control animals and those vaccinated with neat vaccine in heparinized vials (BD Vacutainer, USA) for isolation of PBMCs and separation of plasma. The blood samples were collected on days 0, 5, 10, 15, 21 post-vaccination and on days 3, 5 and 12 post-challenge.

Liquid phase blocking ELISA (LPBE)

LPBE was used to quantify protective antibody level against FMD following vaccination (Hamblin *et al*., 1986). To coat 96 well ELISA plates, 50 µl of specific anti-FMDV antibody (Type A, O and Asia 1), was added in each well, plates were incubated at 37°C for 1 h and kept overnight at 4°C. Two-fold dilutions (starting from 1:32 to 1:256) of test plasma samples were made in the Perspex plates for each type of FMDV and equal volume (75 µl) of diluted FMDV antigen was added and incubated overnight at 4°C, this is known as antigen-antibody mixture. Antigen-antibody mixture (50 µl) was added in duplicate wells of coated plates (brought to room temperature) and washed. Tracing sample (50 µl) for each Type of FMDV was added into wells; plates were incubated for 1 h and washed. 50 µl of anti-Guinea Pig HRPO conjugate was added and plates were incubated at 37°C for 1 h. After the wash, 50 µl of substrate was added and plates were incubated at 37°C for 15 minutes. Positive, negative and background controls were also kept. Reaction was stopped with 50 µl of 1 M H_2SO_4. ELISA reader (Tecan Spectra, Germany) at wavelength of 492 nm (reference 620 nm) was used to read plates.

3AB NSP antibody ELISA

3AB NSP ELISA test was performed as per published literature (Mohapatra *et al*., 2010). In the test, anti-NSP (3AB) antibody present in the plasma samples of infected animals is measured against purified recombinant 3AB antigen. For coating 50 µl of diluted 3AB protein was added in each well of the 96 well ELISA plates and incubated overnight at 4°C. Before use plates were kept at

37°C for 15 minutes. Test and control samples were diluted (1:20) in Perspex plates. After each coated plate was washed thrice, diluted plasma samples (100 µl) were added in duplicate wells and plates were incubated for 1 h at 37°C with intermittent shaking. Positive, negative and background controls were also kept. After washing, 50 µl of anti-bovine HRPO conjugate (1:2000) was added in each well and incubated for 1 h at 37°C with intermittent shaking. After the wash, 50 µl of freshly prepared H_2O_2 (Merck, Germany) activated Orthophenylenediamine dihydrochloride (OPD) (Sigma, USA) substrate solution was added in each well and plates were incubated for 15 minutes at 37°C without shaking. Reaction was stopped with 50 µl of stop solution (1 M H_2SO_4). ELISA reader (Tecan Spectra, Germany) at wavelength of 492 nm (reference 620 nm) was used to read plates.

Isolation of peripheral blood mononuclear cells (PBMCs)

Blood sample (3 ml) was carefully and slowly pipetted on inner-side of wall of 15 ml sterile pyrogen free conical polypropylene centrifuge tubes containing 3 ml of Histopaque-1077 solution (Sigma-Aldrich™, USA) (Boyum, 1968). After balancing, the tubes were centrifuged at 400 x g for 30 minutes at room temperature. On centrifugation, whitish circular band of PBMCs observed in between upper plasma and lower red cellular layer was collected and transferred to another 15 ml centrifuge tube with 5 ml of RPMI-1640 (Sigma, USA). The mix was centrifuged at room temperature for 10 minutes at 250 x g. Resulting cell pellet was again washed as above and the final cell pellet was diluted in 200 µl of dilution medium of RPMI-1640 with 5% FBS (HyClone, USA). Fifty microlitres each of the diluted cells were transferred to four 1.5 ml tubes. To each tubes 500 µl of RNA protect solution (Qiagen, Germany) was added. The tubes were labeled and stored at -70°C until further use.

Isolation of total RNA and first strand cDNA synthesis

The tubes containing cells preserved in 500 µl of RNA protect cell reagent were were thawed at room temperature (22 to 25°C). Mixture was centrifuged at 5000 g for 5 minutes. Resulting supernatant was discarded carefully. The cell pellet was loosened by tapping the sides of the tubes and RLT buffer from RNeasy mini kit (Qiagen, Germany) was added. Total RNA was extracted using RNeasy mini kit. The total RNA was measured using Nano Drop 1000 (Thermo Scientific Spectrophotometer, USA). A total of 500 ng of RNA was used to synthesize the first strand cDNA by using MMLV-RT (200 U/µl) and 1 µg of Oligo dT_{15} according to standard protocol in the total reaction volume of 25 µl. Tubes containing cDNA were kept at - 80°C.

Two step quantitative Real time-polymerase chain reaction (RT-PCR) for measurement of IFN-γ specific mRNA

Oligonucleotide synthesized primers (Standard, 0.02 µmol) for IFN-γ (Product size -171 bp; Forward-5'-GAA TGG CAG CTC TGA GAA ACT GGA-3', Reverse-5'-CGG CCT CGA AAG AGA TTC TGA CTT -3' (Lahmers *et al*., 2006) and β-actin (Product size - 153 bp; Forward-5'-CGA TGA AGA TCA ART CAT TGC -3' and Reverse-5'- AAG CAT TTG CGG TGG AC -3') were obtained commercially (Metabion GmBH, Germany). RT-PCR was performed using a 7500 real-time PCR system as per manufacturer's instructions (AB Applied Biosystems, USA). Quantitation by comparative CT (ΔΔ) type of

experiment was employed in a total reaction volume of 20 µl. Of QuantiTect® SYBR® Green RT-PCR kit (Qiagen, Germany), only 2x Quanti Tect SYBR Green RT-PCR Master Mix was used in the reaction mixture. 1 µl of cDNA was added into each well with 19 µl of reaction mixture (10 µl of 2x Quanti Tect SYBR Green RT-PCR Master Mix, 0.8 µl of each primer (total concentration of 4 pmole of each primer in the reaction), 7.4 µl of RNase free water) in the MicroAmp™ optical 96-well plate (AB Applied Biosystems, Singapore). cDNA sample from young calves at 10 DPV or 0 DPI was used as a reference standard. β-actin was used as endogenous control. Negative controls were also kept in the experiment. After the short spin (1200 g for 3 minutes) the plate was incubated under the following conditions: initial activation of HotStar Taq DNA polymerase at 95°C for 15 minutes, followed by 45 cycles of 94°C for 15 second as denaturation, 55°C for 30 second for annealing and 72°C for 30 second extension. Melt curve analysis was performed as per instruments standard. Gene expression data generated by the software was recorded.

RESULTS AND DISCUSSION

Results of LPBE on plasma samples were shown in the Table 1. (DPV-Days post vaccination, DPC-Days post challenge, DPI-Days post infection, Vaccinated and challenged: 209, 211, 212, 257, 1004, Unvaccinated control and infected: 202, 1022).

Critical view of LPBE results indicate, highest FMDV antibody titers on 15 DPV in 4 out of 5 vaccinated calves. 3AB NSP ELISA showed that calves were free of FMDV infection at the time and during the period of vaccination. All seven calves were positive for presence of anti-NSP antibodies on 12 DPC. Vaccinated calves were protected against FMD up on challenge as there was no clinical signs of disease were observed. In control calves on 3 DPI typical signs of FMDV including lesions on tongue were noticed after clinical examination. Prevalence of cytokines in the blood is one of the first indications of infection (Flint *et al.*, 2004). Cytokines including interferons are implicated in FMDV pathogenesis, immunity and protection (Chinsangaram *et al.*, 1999; Parida *et al.*, 2006; Moraes *et al.*, 2007; Nfon *et al.*, 2008; Zhang *et al.*, 2009). So in the present investigation efforts have been made to understand IFN-γ response elicited after FMDV infection and vaccination. Real-time PCR based identification and measurement of cytokine mRNA in whole blood, PBMCs, white blood cells, dendritic cells and sputum have been described. A few reports are available on in-vivo cytokine response after FMDV infection and vaccination in experimental models of bovines and swine (Barnett *et al.*, 2002; Bautista *et al.*, 2005). In this study in-vivo effects of FMDV vaccination and infection on expression of IFN-γ mRNA in PBMCs isolated from naïve cattle calves was analyzed.

In the study, PBMCs were isolated from blood samples collected at different intervals from individual crossbred cattle calves (FMDV uninfected, apparently healthy and unvaccinated with no history of FMD) from FMDV Vaccine Potency testing. Fixed quantity of RNA was used to prepare cDNA from PBMCs isolated from blood. RNAprotect solution was used to protect RNA expression levels in PBMCs till its transportation to laboratory and extraction of RNA. Probably such approach has never been used to quantify IFN-γ expression after FMDV infection and vaccination. SYBR Green based two-step real-time PCR (comparative CT (ΔΔ)

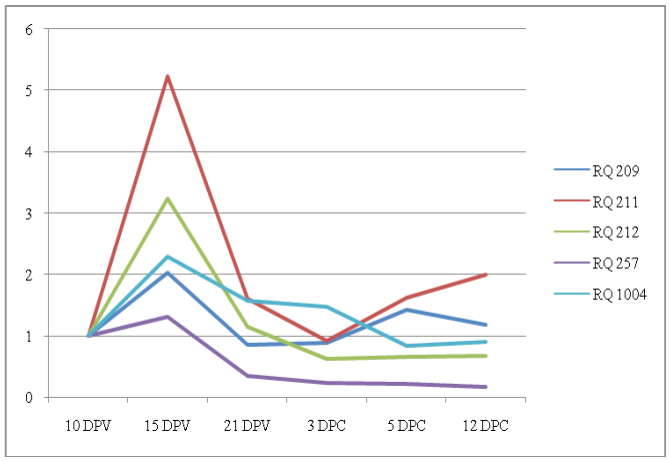

Figure 1. IFN-γ mRNA level in vaccinated and challenged bovines. (DPV-Days post vaccination, DPC-Days post challenge, RQ-Relative quantification, Vaccinated and challenged: 209, 211, 212, 257, 1004).

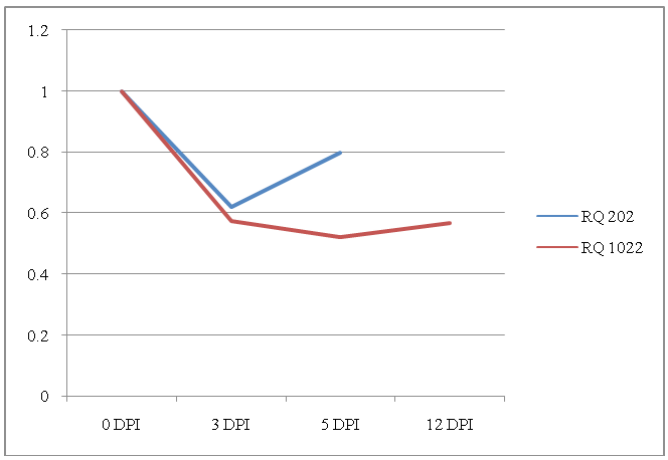

Figure 2. IFN-γ mRNA level in infected bovines. (DPI-Days post infection, RQ-Relative quantification, Unvaccinated control and infected: 202, 1022).

type of experiment) was employed using β-actin as endogenous control. IFN-γ mRNA expression data generated by the machine as RQ values were plotted in the format of the chart. cDNA prepared from PBMCs collected on 10 DPV (first isolated PBMCs) was used as reference sample. Increase in mRNA expression level of IFN-γ after 15 DPV was found unequivocally in all five vaccinated calves (more than two-fold increase in 4 out of 5 calves) (Figure 1). LPBE test results also revealed highest humoral antibody response on 15 DPV (Table 1). Increased IFN-γ mRNA expression in buffaloes on 21 days of monovalent Type O FMDV vaccination was reported except its relation to humoral response (Mingala *et al.*, 2009). Up on challenge of vaccinated calves in the present experiment, there were no fixed trends of increase or decrease in IFN-γ mRNA expression. Though after FMDV infection, in control unvaccinated cattle calves there was decrease in IFN-γ mRNA expression (Figure 2). Immune response to FMDV is affected by host (species, breed, age, health, physiological states and FMD immune status and others) and viral variables (dose, route, volume, viral strain and others) (Doel, 1996). Similarly, mRNA expression levels of IFN-γ and other cytokines may also be influenced by the above-mentioned factors. Cytokine response in the respiratory tract and blood on FMDV infection in cattle need to be studied in order to devise a test helpful in early diagnosis of the infection before onset of clinical sickness. In future, protein based tests and tests targeting specific cell population in PBMCs will be employed. To sum up, in the present study in bovine cattle, IFN-γ mRNA level was highest on 15 DPV after monovalent Type O FMDV vaccination and decreased thereafter. Up on experimental challenge with live FMDV in unvaccinated cattle calves, there was a two-fold decrease in IFN-γ mRNA level.

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Table 1. Liquid phase blocking enzyme linked immunosorbent assay results indicative of antibody titres.

Animal	0 DPV	5 DPV	10 DPV	15 DPV	21 DPV	3 DPC/I	5 DPC/I	12 DPC/I
209	<1.50	<1.50	1.68	1.98	1.98	1.98	2.28	>2.40
211	<1.50	<1.50	1.68	1.68	1.68	1.68	2.28	>2.40
212	<1.50	<1.50	<1.50	1.68	1.68	1.68	2.28	>2.40
257	<1.50	<1.50	1.98	>2.40	>2.40	>2.40	>2.40	>2.40
1004	<1.50	<1.50	<1.50	1.98	1.98	1.98	2.28	>2.40
202	<1.50	<1.50	<1.50	<1.50	<1.50	<1.50	2.28	>2.40
1022	<1.50	<1.50	<1.50	<1.50	<1.50	<1.50	1.68	>2.40

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