TOLL LIKE RECEPTOR 7 MESSENGER RIBONUCLEIC ACID EXPRESSION LEVELS IN DAIRY ANIMALS IN AN OUTBREAK OF FOOT-AND-MOUTH DISEASE

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ABSTRACT

Foot-and-Mouth disease (FMD) is an economically most important disease of dairy animals in India. It is caused by Foot-and-Mouth disease virus (FMDV) an under the family Picornaviridae. Pathogenesis and immune responses elicited by FMDV in the processes of infection and vaccination are not completely understood. Toll like receptor 7 (TLR7) is related to inflammation. The present study is aimed at evaluating messenger ribonucleic acid (mRNA) expression of TLR7 in peripheral blood mononuclear cells isolated from dairy animals experiencing an outbreak of FMD in real-time polymerase chain reaction test. TLR mRNA expression level on in an outbreak of FMD is reported probably for the first time. The mRNA expession levels of TLR7 were found at lower side in dairy cattle cows that showed clinical FMD twice as compared to in-contact apparently healthy dairy cattle cows. The present investigation will help in understanding the immune response of bovines against FMD in an outbreak of FMD.

Keywords: buffalo, *Bubalus bubalis*, dairy, bovines, FMD, TLR7, mRNA, Real-time, PCR

INTRODUCTION

Foot-and-Mouth disease (FMD) is caused by Foot-and-Mouth disease virus (FMDV) an Aphthovirus classified in the family *Picornaviridae*. According to World organization for Animal Health FMD is a notifiable disease of ruminants. Ministry of Agriculture in its 19th Livestock census reports a total cattle and buffalo population of 300 million in India. Losses due to FMD in the country have been estimated at Rs. 20,000 crores. There are seven types of FMDV prevalent across the globe but in India at present only three types exist (O, A, Asia 1). As per Project Directorate on Foot-and-Mouth Disease, the majority of outbreaks are due to type O followed by type Asia 1 and type A. Pathogenesis and the immune responses elicited by FMDV in various livestock species are not completely understood as per Summerfield *et al*. (2009); Zhang *et al*. (2009). The present investigation was undertaken to study mRNA expression levels of TLR7 in peripheral blood mononuclear cells isolated from dairy cattle cows experiencing an outbreak of FMD.

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MATERIALS AND METHODS

Whole blood samples which were collected in heparinated vacutainers (BD Vacutainer® with anticoagulant Sodium heparin 143 USP U) from 20 Holstein Friesian crossbred dairy cattle (*Bos taurus* x *Bos indicus*) in an outbreak of FMD. The animals showing no clinical signs were kept in the group of in-contact apparently healthy and those with clinical signs in the group of clinically FMDV infected. Most of the bovines received a dose of commercially available trivalent inactivated vaccine against FMD (Vaccination status of two animals not known). Serum samples were also extracted. Clinical samples from the current outbreak when used in FMDV typing test in the laboratory at Project Directorate on Foot-and-Mouth Disease (PDFMD) indicated implication of type A FMDV infection. Clinically affected cattle showed loss in milk production, foot and mouth lesions and profuse salivation. These cows received antibiotic treatment to check secondary bacterial infections. Liquid phase blocking enzyme linked immunoassay (LPBE) test is routinely performed in the laboratory to quantify protective antibody level against FMD in the animals following vaccination against FMD. Log_{10} values were recorded for interpretation of LPBE results (<1.8 considered unprotective).

Differentiation between infected and vaccinated animals (DIVA) test developed at PDFMD based on recombinant non-structural polyprotein 3AB used to detect infected animals. Oligonucleotide synthesized primers (Standard, 0.02 µmol) for TLR7 were procured from Metabion GmBH (Germany) (Forward-5'-ACT CCT TGG GGC TAG ATG GT-3' and Reverse-5'-GCT GGA GAG ATG CCT GCT AT-3'; 180 bp, Menzies *et al*. (2005). Primers for β-actin (Forward-5'-CGA TGA AGA TCA ART CAT TGC-3' and Reverse-5'-AAG CAT TTG CGG TGG AC-3'; 153 bp) were available at PDFMD and used in the investigation. Blood samples were brought to the laboratory at +4°C after 4 h of journey and kept overnight at 4°C until peripheral blood mononuclear cells (PBMCs) isolation as per Boyum (1968). Diluted blood sample (with equal amount of RPMI-1640 with 5% FBS) was carefully and slowly pipetted on the inner-side of the wall of the 15 ml sterile pyrogen free conical polypropylene centrifuge tubes with already added Histopaque®-1077 solution. Though few undiluted blood samples were used to isolate PBMCs. After balancing, tubes were centrifuged at 400 x g for 30 minutes at room temperature. On centrifugation, whitish circular bands of PBMCs were observed in between upper plasma and lower red cellular layer. Resulting plasma samples were collected and kept at -70°C. Band of PBMCs was transferred with the help of glass Pasteur pipette to another 15 ml centrifuge tube with 5 ml of RPMI-1640 and centrifuged at room temperature for 10 minutes at 250 x g. The resulting cell pellet was again washed as per the above. Pelleted cells were directly used for RNA extraction. Total RNA was extracted using RNeasy kit as per the manufacturer (Qiagen). RNA was measured using Nano Drop 1000. One step real-time polymerase chain reaction (PCR) was performed using a 7500 real-time PCR system (AB Applied Biosystems, USA). Quantitation by comparative CT (ΔΔ) type of experiment was employed in a total reaction volume of 20 µl.

QuantiTect® SYBR® Green RT-PCR kit was used to perform the experiments. 20 ng of total RNA was used in the reaction to study expression level of TLR7. 2 µl of diluted total RNA was added into 18 µl of reaction mixture (10 µl of 2x Quanti Tect SYBR Green RT-PCR Master Mix, 0.2

µl of Quanti Tect RT Mix, 0.4 µl of each primer (total concentration of 2 pmole of each primer in the reaction), 7.0 µl of RNase free water) in the MicroAmp™ optical 96-well plate. One of the RNA sample from in-contact healthy cow 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 RT enzyme at 50°C for 30 minutes, followed by denaturation at 95°C for 15 minutes, thereafter 50 cycles of 94°C for 15 seconds as denaturation, 55°C for 30 seconds for annealing and 72°C for 30 seconds extension. Melt curve analysis was performed as per instruments standard. Gene expression data generated by the software was recorded and analyzed.

RESULTS AND DISCUSSION

Self or non-self recognition of nucleic acids in viral infection is relied on several pattern recognition receptors (PRRs) including toll-like receptors (TLRs). Double stranded RNA with the help of TLR7 activates immune cells to secrete cytokines. Koyama *et al*. (2008) have described role of TLR7 along with other TLRs in viral infections. Hence TLR7 mRNA expression levels in dairy cattle cows are studied in the present investigation. Konnai *et al*. (2003); Zhang *et al*. (2006) analysed bovine cytokine genes and toll like receptors by real-time polymerase chain reaction. Mingala *et al*. (2009) reported quantification of cytokines in buffalo on vaccination against FMD but there are very few reports on the study of *in-vivo* cytokine and toll like receptor's response to FMDV infection in cattle. But no reports are available on investigation of bovines in an outbreak of FMD. This will and may be the first report describing the mRNA expression levels for TLR7 in dairy animals in an outbreak of FMD. A total of 20 total RNA samples extracted from PBMCs from dairy animals in an outbreak of FMD with different history of FMDV infection were investigated. SYBR Green based one step real-time PCR (comparative CT (ΔΔ) type of experiment) was employed to study mRNA expression levels of TLR7 by the method of Livak and Schmittgen (2001).

History revealed introduction of few animals in the herd (R-54, R-57). To assess protective antibody levels in these dairy cattle cows, representative samples were tested in liquid phase blocking enzyme linked immunoassay (LPBE). Serum samples from these cows indicated titers upto and more than 1.8. Sandwich enyme linked immunoassay (ELISA) testing at PDFMD on clinical samples collected from the dairy cattle cows during the present outbreak by the method of Mohapatra *et al*. (2007) has pointed out the outbreak to be of type A FMDV. Differentiation between infected and vaccinated animals (DIVA) test developed by Mohapatra *et al*. (2010) was employed in the study. Most of the animals irrespective of their group indicated presence of NSP (titers more than 0.3) in DIVA test.

Comparison in mRNA levels in betweeen dairy cattle cows in real-time PCR was understood on forming four different groups according to their current status of clinical sickness on the day of blood collection; (1) Five in-contact apparently healthy cows -A-12, A-29, B-1, M-53, M-54, (2) Six of the cows in which clinical FMD was noticed within 10 days -B-4, H-65, R-54, R-57, T-61, T-69 (3) Six cows in which clinical FMD was noticed only once but before 1 to 4 months -B-6, B-10, B-17, B-40, M-57, T-70, and (4) Three cows in

which clinical FMD was noticed twice (re-infected) -B-19, M-51, M-56. RQ log10 values of dairy cattle cows are compared by keeping one of the in-contact apparently healthy cows as a control in the experiment. Results of real-time PCR analysis were depicted in the Figure 1. and Table 1.Table 1: Results of real-time polymerase chain reaction test for the study of TLR7 messenger ribonucleic acid expression levels in peripheral blood mononuclear cells from dairy animals in an outbreak of Foot-and-Mouth disease. Melt curve analysis for TLR7 and β-actin were depicted in Figure 2. Role of cytokines in response to inflammatory diseases and viral infections is well described. The mRNA expession levels of TLR7 were found at lower side in dairy cattle cows that showed clinical FMD twice

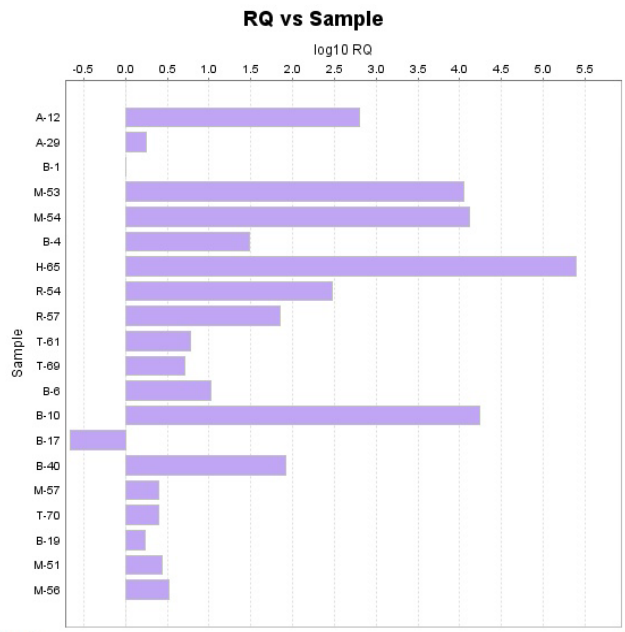

Figure 1. TLR7 mRNA expression levels in dairy bovines in an outbreak of Foot-and-Mouth disease.

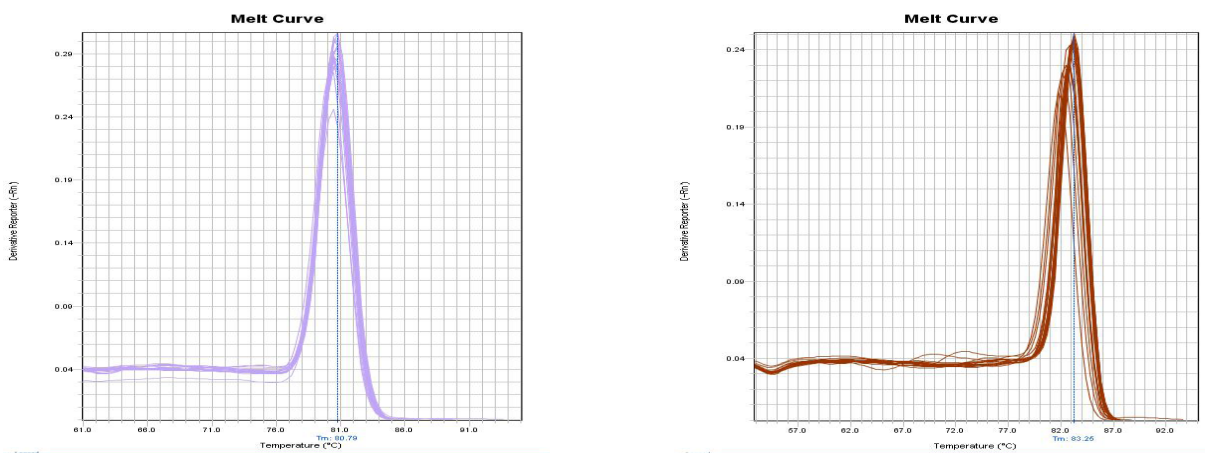

Figure 2. Melt curve analysis of TLR7 and β-actin.

Table 1. Results of real-time polymerase chain reaction test for the study of TLR7 messenger ribonucleic acid expression levels in peripheral blood mononuclear cells from dairy animals in an outbreak of Foot-and-Mouth disease.

Groups of dairy cows depending on history	Identity	RQ values	Average RQ value for groups	Log10 RQ values	Average Log10 RQ value for groups	Comparison of TLR7 mRNA expression levels
1) In contact-apparently healthy	A-12	626.979	4998.735	2.797	2.243	Increased expression level compared to groups 3 and 4
	A-29	1.775		0.249		
	B-1	1		0		
	M-53	11239.78		4.051		
	M-54	13124.14		4.118		
2) Clinical FMD was noticed within 10 days	B-4	30.849	40882.94	1.489	2.117833	
	H-65	244883.4		5.389		
	R-54	301.039		2.479		
	R-57	71.11		1.852		
	T-61	6.07		0.783		
	T-69	5.183		0.715		
3) Clinical FMD was noticed only once but before 1 to 4 months	B-6	10.654	2911.519	1.028	1.22033	Expression level less than groups 1 and 2 but greater than group 4
	B-10	17371.2		4.24		
	B-17	0.221		-0.655		
	B-40	82.044		1.914		
	M-57	2.534		0.404		
	T-70	2.459		0.391		
4) Clinical FMD was noticed twice/re-infected	B-19	1.693	2.589333	0.229	0.397	Lowest expression level
	M-51	2.777		0.444		
	M-56	3.298		0.518		

as compared to in-contact apparently healthy dairy cattle cows and other two groups (cows in which clinical infection was noticed within 10 days and cows in which clinical FMD was noticed only once but before 1-4 months of sample collection). The present investigation will help in understanding the immune response of bovines against FMD in an outbreak of FMD. Further investigations in the field outbreaks are required to understand role of TLR7 in livestock with respect to FMDV infection vis-à-vis protection.

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