

Profiling of bovine toll like receptors (TLRs) in foot and mouth disease vaccinated cattle

RAJEEV RANJAN¹, JITENDRA KUMAR BISWAL², AJAY KUMAR SHARMA³,
JYOTI MISRI⁴ and BRAMHADEV PATTNAIK⁵

ICAR-Project Directorate on Foot-and-Mouth Disease, Mukteshwar, Nainital, Uttarakhand 263 138 India

Received: 16 July 2015; Accepted: 21 August 2015

ABSTRACT

Foot and mouth disease virus (FMDV) elicits acute humoral antibody response in both infected and vaccinated animals. Toll like receptors (TLRs) are type 1 transmembrane proteins expressed in almost all cell types and activate the innate immune system. The current study was performed to evaluate expression profiling of bovine TLRs like TLR 2, TLR 3, TLR 7, TLR 8 and TLR 10, in response to FMD inactivated vaccine using quantitative real-time RT-PCR technique. Blood samples were collected from control, test group 1 and test group 2, at 0, 14th and 21st days post-vaccination (dpv). The mRNA abundance of these target genes was calibrated with a housekeeping gene (18 S) and expressed as fold over expression of the TLRs genes in bovine over the 0th dpv as control. On 0 day, expression of all TLRs did not vary significantly. The expression of TLR2 and TLR3 genes significantly increased in both test group 1 and 2 after 14th day and 21st DPV but expression of other TLRs increase in test groups 1 and 2 did not differ significantly. Expression of TLR2 and TLR3 genes considerably increased in test group 1 and 2 but expression of these genes were more in test group 1 as compared to test group 2. From preliminary findings, if there is inclusion of TLR2 and TLR 3 agonist in vaccine, it may enhance the innate immunity of animals and helps in clearing of virus and may prevent establishment of infection.

Key words: Cattle, FMDV, Real-time RT-PCR, TLRs profile, Vaccine

Foot-and-mouth disease virus (FMDV), the etiological agent of foot-and-mouth disease (FMD)— a highly contagious and economically important viral disease of both cloven footed and wild animals (Alexandersen *et al.* 2003), is icosahedral symmetry, non-enveloped, a positive strand RNA virus, belonging to the genus *Aphthovirus*, family Picornaviridae (Belsham 1993). Serologically and immunologically, FMDV is present in 7 serotypes viz. O, A, C, Asia 1, SAT-1, -2, -3 (Robson *et al.* 1977), which are further sub-divided into distinct topotypes/genotypes based on phylogenetic analysis (Knowles and Samuel 2003, Mohapatra *et al.* 2011). FMD is endemic in India with serotypes O, A and Asia 1 (Biswal *et al.* 2012, Ranjan *et al.* 2014). FMDV elicits acute humoral antibody responses in infected or vaccinated animals, which have been considered to be the most important protective factor against FMD infection. There are increasing numbers of studies discussing about innate immune responses during FMDV infection or vaccination in recent years particularly in

endemic country like India. The main goal of vaccination is to induce immunologic protection from infectious diseases of bacterial, viral and parasitic origin. Vaccination regimes are a powerful approach to protect our animal populations against microbial diseases. However, purpose of vaccination regimes requires a comprehensive knowledge about all potential vaccination effects.

Vaccination, the most important tool for control of FMD in endemic country where the slaughter of animals is not practiced, leads to changes in expression of genes which directly or indirectly elicit the immune system by activating the immune cells. The importance of innate immune defenses for the control of FMDV, principally during in early infection (Summerfield 2009). FMDV can escape immune responses through its ability to shut down cellular protein synthesis, including IFN type 1, in susceptible epithelial cells.

The mammalian immune system consists of two different arms—innate and adaptive immunity—and cooperative interactions of these 2 arms are required for elimination of different pathogens with the utmost efficiency. Toll like receptors (TLR) are type 1 transmembrane proteins and activate innate immune system upon sensing pathogen associated molecular patterns (PAMPs). Recognition of pathogens by innate immune system is mediated through

Present address: ¹Scientist (drrajraj@gmail.com), ²Scientist (jkubiswal@gmail.com), ⁵pattnaikb@gmail.com, ICAR-Project Directorate on FMD, ³Principal Scientist (sharmaak62@gmail.com), TAH, IVRI Campus Mukteshwar. ⁴Principal Scientist, (jmisri.icar@gmail.com), Indian Council of Agricultural Research, Krishi Bhawan, New Delhi.

pattern recognition receptor (PRR) such as toll-like receptors (TLR) (Takeda and Akira 2005). Until now, 13 TLRs have been described in mice, 11 in human and 10 in bovine. The TLR family members can be conveniently divided into 2 subpopulations with regard to their cellular localization. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed exclusively on the cell surface and recognize microbial membrane components while TLR3, TLR7, TLR8 and TLR9 are localized in intracellular vesicles such as the endosome or lysosome and the endoplasmic reticulum (ER) (Latz *et al.* 2004, Nishiya *et al.* 2005). Intracellular TLRs that sense viral nucleic acids include TLR3 (double stranded RNA), TLR7 and TLR8 (single stranded RNA) and TLR9 (CpG motifs in DNA) (Kawai and Akira 2007). These intracellular TLRs appear to be sensors of foreign nucleic acids and trigger anti-viral innate immune responses. Up-regulation of TLR2 is observed in both virus infected cells as well as in DNA vaccine transfected cells in which FMDV antigen was a common factor. In FMDV infected or vaccinated animals, an increased level of type I IFN was reported (Cheng 2007). The vaccine DNA successfully reaching nucleus may express the encoded antigen, which in turn processed as natural viral antigen in endosomes leading to increased expression of TLR 2 (Gaikwad 2012). TLR3 messenger RNA (mRNA) is detected in conventional dendritic cells (cDCs) and macrophages as well as by non-immune cells including fibroblasts and epithelial cells, and strong expression of TLR3 is found in CD8a+ DCs with high phagocytic activity for apoptotic bodies of virus-infected or dsRNA loaded cells. This allows dsRNA to gain access to TLR3 within cells and activate the signaling cascade to produce IL-12 p40 and IFN γ , suggesting a role of TLR3 in triggering cross-presentation, which processes exogenous antigens within the MHC class I pathway (Schulz *et al.* 2005). TLR7 was originally identified to recognize imidazoquinoline derivatives and guanine analogues, which have anti-viral (Hemmi 2002). It is highly expressed on plasmacytoid dendritic cells (pDCs), a subset of DCs with a plasmacytoid morphology that are unique in their capacity to rapidly secrete vast amounts of type I IFN in response to viral infection (Gilliet *et al.* 2008). Viruses are likely to be endocytosed and retained in the endosomal compartments, where the viral particles are subsequently degraded,

allowing the viral RNA to engage with TLR7.

Vaccination is an extremely powerful tool for preventing infectious diseases. Eradication of smallpox and the dramatic reduction in polio and measles throughout the world represent the most significant success of vaccination till date. Moreover, innate response represents a promising strategy to develop improved emergency vaccine capable of rapidly establishing a protective status. It is clear that Toll-like receptors (TLRs) and their signaling pathways play a role in the innate immune response (Doyle *et al.* 2003). There is paucity of information available about the innate immune response to FMDV and differential expression profile of TLRs against FMD inactivated vaccine in cattle under natural condition. Hence, the current study was performed to evaluate expression profiling of bovine TLRs like TLR 2, TLR 3, TLR 7, TLR 8 and TLR 10, in response to FMD inactivated vaccine using quantitative real-time PCR technique in cattle.

MATERIALS AND METHODS

Animals, vaccination and blood collection: The present study was carried out at the experimental cattle herd at Mukteshwar, which is in the Kumaon ranges of Himalaya (7,500 feet above mean sea level). All were crossbred HF cattle and regularly vaccinated at six months interval. Naïve calves (12) and adult animals (6) were used in the present study. All animals were negative for brucellosis, tuberculosis, and John's disease, and were dewormed before vaccination. Animals were categorized into three groups, such as, six naïve calves (control group), six primo vaccinate calves (test group 1) and the other six revaccinated adult animals (test group 2). The test group 1 and 2 animals were vaccinated intramuscularly with 2 ml of trivalent FMDV inactivated vaccine. Blood samples were collected from the jugular vein into sodium heparin Vacutainer (BD Vacutainer) at 0th, 14th and 21st days post vaccination and immediately processed for further studies.

RNA extraction: Total RNA was extracted from whole blood using RNA isolation kit according to the manufacturer's instruction. The extracted RNA was further purified and treated with DNaseI using RNeasy kit and was quantified by spectrophotometer considering the OD260/OD280 ratio, which was 1.8–2.0 for all the samples. The

Table 1. Primer sequence and other details of real-time PCR performed for expression analysis of different TLRs and internal control 18S genes of bovine

S. No.	Gene	Forward primer (5' - 3')	Reverse primer (5' - 3')	Amp size (bp)	References
1.	TLR 2	ACGACGCCTTTGTGTCCTAC	CCGAAAGCACAAAGATGGTT	192	Menzie's and Ingham (2006)
2.	TLR 3	GAGGCAGGTGCTCTTGAAC	GCTGAATTTCTGGACCCAAG	329	
3.	TLR 7	ACTCCTTGGGGCTAGATGGT	GCTGGAGAGATGCTGCTAT	180	
4.	TLR 8	TCCACATCCCAGACTTTCTACGA	GGTCCCAATCCCTTTCTCTA	150	
5.	TLR 10	TCTGCCTGGGTGAAGTATGA	AATGGCACCATTGAGTCTGG	190	Lahmers <i>et al.</i> (2006)
6.	18 S	CTGAGAAGACGGTCAACTTGACT	TCCGTTAATGATCCTTCCGAGGT	90	

total RNA yields in each sample were diluted to 10 ng/ μ l for further analysis. Total RNA preparations were stored at -80°C till further use.

Primers: Sequences of reported primers of TLR 2, TLR 3, TLR 7, TLR 8, TLR 10 and the 18 S (as an internal control) genes were used for quantification of different TLRs by real time RT-PCR. Details of real-time PCR primers for above genes are shown in Table 1.

Real-time RT-PCR: Real-time RT-PCR was performed using RT-PCR kit. It was carried out by a standard protocol recommended by the manufacturer with 2 μ l (10ng/ μ l) of total RNA, which was added to an 18 μ l reaction mixture including 0.2 μ mol of each primer, 10 μ l of 2 $\times$ RT master mix, 0.16 μ l RT enzyme and 7.44 μ l of nuclease free water. The mixture was run in a ABI Real Time PCR machine 7500 under the following conditions: at 50°C for 30 min as RT, at 95°C for 15 min as the denaturation program, followed by 40 cycles of 94°C for 15 sec, annealing at 56°C for 30 sec, 72°C for 30 sec and each of optimal fluorescence measurement temperature for 1 sec as the amplification and quantification programs, 60–95°C with a heating rate of 0.5°C/sec as the melting curve program and finally cooling to 40°C as the cooling program. Two replicates of each sample were placed.

For each TLR gene, it was confirmed that the efficiency of amplification was similar with 18 S gene (data not shown), permitting assessment of fold change of genes with the formula $2^{-\Delta\Delta CT}$. Therefore, fold change of mRNA was determined from the threshold cycle (C_T) values normalized for 18S gene expression and then normalized to the value derived from non vaccinated animals at 0 days (Schmittgen and Livak 2008).

Statistical analysis: The statistical significance of TLR genes expression between blood samplings was tested by Student's two-tailed t-test, assuming equal variances. Differences between each of them were considered significant if probability values of $P < 0.05$ were obtained. The statistical tests were performed using win stat software.

RESULTS AND DISCUSSION

Blood samples taken from all 36 groups, such as, six naïve calves (control group), six primo vaccinate calves (test group 1) and the other six revaccinated adult animals (test group 2), at 0th, 14th and 21st days post-vaccination (dpv) against FMD inactivated vaccine were analyzed for expression of TLR gene by real time RT-PCR. There was differential expression of TLR 2, TLR 3, TLR 7, TLR 8 and TLR10 (Fig. 1) recorded in this study. The mRNA abundance of these target genes was calibrated with that of a housekeeping gene (18 S) and expressed as fold over expression of the TLR genes in bovine over the 0 day post vaccination control. Melting point of each TLR and housekeeping gene was estimated for specific amplification (Fig. 2). After optimization of melt curve, quantification of each TLR was carried out. Differential expression of TLRs 2, 3, 7, 8, and 10 were neither upregulated nor down regulated in control group after 0, 14th and 21st dpi

(Fig. 1). In primo vaccinate calves (test group1), all TLRs were upregulated after 14th and 21st dpv, but significant difference (at $P < 0.05$) was observed only in TLR 2 and 3 (Fig. 1). Similarly in revaccinated adult animals (test group 2) also, all TLRs were upregulated while there was significant difference (at $P < 0.05$) only recorded in TLR 2 and 3 after 14th and 21st dpv (Fig. 1). On 0 day, expression of TLR 2, 3 and 7 was higher, while TLR 8 and 10 much less in test group 2 on 0 dpv; but expression of TLRs did not vary significantly ($P < 0.05$). The expression of TLR genes either up regulated or down regulated in test group 2 and it could be due to prior vaccine effect. On 14th dpv, there was

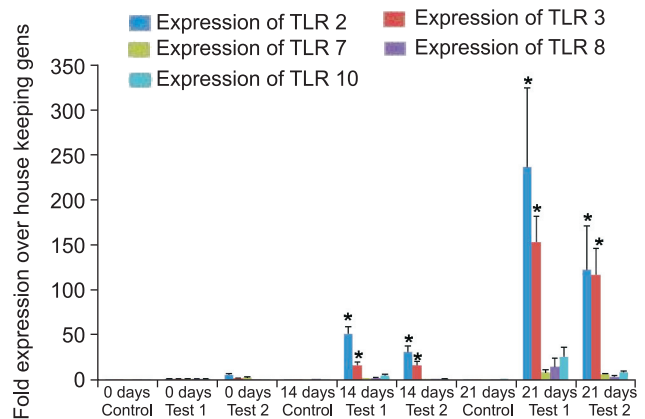


Fig. 1. Comprehensive representation differential expression of TLRs 2, 3, 7, 8 and 10 genes against FMD inactivated vaccine on different days post vaccination (dpv) analyzed by real time quantitative RT-PCR. Fold over expression of the TLRs gene in FMD inactivated vaccine over the 0 dpv housekeeping gene (18S). * indicates there are significant differences between in expression of TLR 2 and TLR 3 on 14th and 21st dpv.

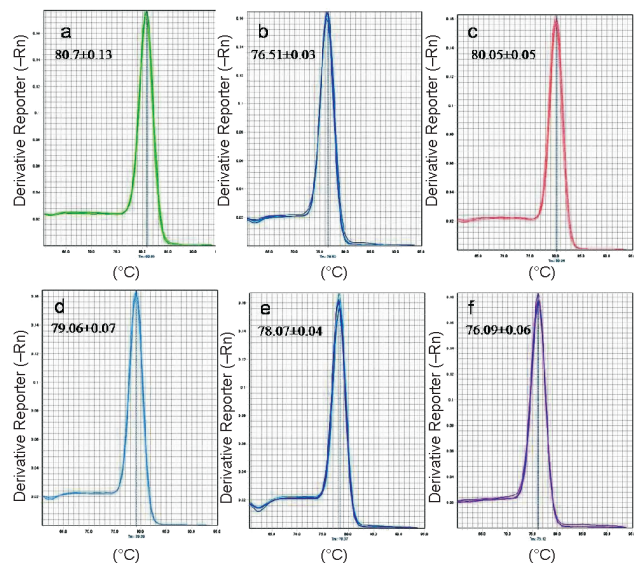


Fig. 2. Sharpness of peak in a melt curve indicating specific amplification of TLRs. Mean $\pm$ SE of T_m is located on each melt curve of different TLRs, viz. a. TLR 2-80.7 $\pm$ 0.13, TLR 3-76.5 $\pm$ 0.03, TLR 7- 80.05 $\pm$ 0.05, TLR 8- 79.06 $\pm$ 0.07, TLR 10- 78.07 $\pm$ 0.04 and 18 S- 76.09 $\pm$ 0.06).

upregulation of all the TLRs but statistically significance different (at $P < 0.05$) was only recorded in TLR 2 and TLR 3. Similar finding was recorded on 21st dpv but on 21st dpv there was increased expression of all the TLRs as compared to 14th dpv. Another finding was that, expression of all the TLRs on 14th and 21st dpv was more in test group 1 as compared to test group 2. Expression of TLR 2 and TLR 3 genes considerably increased ($P < 0.05$) in test group 1 and test group 2, but expression of these two genes was higher in test group 1 compared to test group 2 (Fig. 1), on both 14th and 21st dpv. Expression of other TLRs increased in both test groups 1 and 2 but did not differ significantly ($P < 0.05$). Among TLRs, highest expression was recorded of TLR 2 in test groups 1 and 2, followed by TLR 3 after 14th as well as 21st dpv and varied significantly ($P < 0.05$), while TLR 7, 8 and 10 upregulated after 14th as well as 21st dpv, but statistically did not differ significantly ($P < 0.05$) in both test groups 1 and 2 after 14th and 21st dpv (Fig. 1). Upregulation of TLR 2 could be due to capsid protein of FMD virus especially VP1 and VP3 as this protein acts as TLR 2 agonist (Liang *et al.* 2014). It was reported earlier that TLR 3 agonist activates innate immunity and induces the production of proinflammatory cytokines by activation of NF- κ B through TLR3 signaling pathway (Kawai and Akira 2010). In the current study, we found an upregulation of TLR3 expression in the vaccinated as compared to the unvaccinated control group. Therefore, it could be suggested that oil-adjuvanted inactivated FMD vaccine may stimulate innate immune response through TLR 3 signaling pathways. However, further studies need to be conducted to confirm this suggestion.

In conclusion, the current study was performed to evaluate expression profiling of bovine TLRs like TLR 2, TLR 3, TLR 7, TLR 8 and TLR 10, in response to FMD inactivated vaccine using quantitative real-time RT-PCR technique from control, test group 1 and test group 2, at 0, 14th, and 21st dpv. Expression of TLR 2 and TLR 3 genes considerably increased ($P < 0.05$) in test group 1 and test group 2 but expression of these genes were more in test group 1 as compare to test group 2. It is a powerful and suitable method for fast, accurate, sensitive and cost-effective profiling of bovine TLRs in FMD vaccinated cattle against FMD virus which elicits acute humoral antibody response. In preliminary finding, if there is inclusion of TLR 2 and TLR 3 agonist in FMD vaccine that may enhance the innate immunity of animals and helps in clearing of virus and may prevent establishment of infection.

ACKNOWLEDGMENT

This work was supported by the Indian Council of Agricultural Research under the project number-IXX00086. We are also thankful to dairy persons of IVRI campus, Mukteshwar for their kind support.

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