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Expression and SNP Analysis of Bovine TLR3 Gene in FMD Infected Cattle

Sutopa Das[1]*, Arun Prasad[2], Krishna Sarma[1], Subhash Medhi[3], Sujoy Bose[3] and Manab Deka[3]

[1]AICRP on FMD Regional Centre, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-22, Assam, India.
[2]Ranchi Veterinary College, Birsa Agricultural University, Ranchi, Jharkhand.
[3]Gauhati University, Guwahati, Assam, India.

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Innate immunity is responsible for the initial detection and response to pathogen infection. Because of their role in a host's defense against viruses, Toll Like Receptors (TLRs) are candidates for studies of resistance or susceptibility to viral infection in cattle. TLR3 is involved in the recognition of viral double-stranded RNA which is known to be generated by most viruses during infection as a replication intermediate for ssRNA viruses. Therefore, in the present study, TLR3 was considered as a candidate molecule for studying certain molecular aspects, since the FMDV RNA undergoes a double stranded "replicative form". Effort was made to correlate the expression and mutation in terms of SNPs of TLR3. The severity of the disease was graded as 'more severe', 'less severe' or 'no disease'. TLR3 was found to increase significantly in case of FMDV infected cattle. Out of the total 40 infected samples subjected for TLR3 expression, 14 samples showed marginal expression of TLR3. Among the others, the fold increase in expression varied from moderate to high level. In 'less severe' cases of the disease, TLR3 expression was found to be more and was statistically significant compared to 'more severe' group. For studying the polymorphism in the bovine TLR3 gene, two SNPs (rs42852440 and rs42852439) were targeted and both were mostly prevalent in FMDV cases. From the above study it can be concluded that the expression of TLR3 was correlated with the disease in that in the 'less severe' cases, TLR3 expression was found to be significantly high compared to 'more severe' cases and the presence of SNPs in the coding region was found to exist although there was no statistical correlation with the severity of the disease.

Key words: FMD, TLR3, expression, SNP, disease severity

The innate immune system comprises the cells and mechanisms that defend the host from infection by other organisms, in a non-specific manner. Induction of the antiviral innate immune response depends on recognition of viral components by host pattern- recognition receptors (Akira *et al*., 2006 and Bowie and Haga, 2006). Three classes of receptors, designated retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), Toll-like receptors (TLRs), and nucleotide oligomerization domain (NOD)-like receptors (NLRs), sense viral components, such as double-stranded RNA (dsRNA), single-stranded RNA, and DNA. These classes of pattern recognition receptor (PRR) molecules are expressed in different cell types and different intracellular compartments.

Toll like receptors, said to be the sentries of the innate immune cells, have evolved to detect molecular signatures of invaders with specific conserved structures shared by a large group of microorganisms referred to as pathogen-associated molecular patterns (PAMPs). These molecular structures are essential for replication

* To whom all correspondence should be addressed.
E-mail: d_sutopa@yahoo.com

and/or survival of the microorganism, and were not believed to be ever expressed by the host. Recognition of PAMPs allows the innate immune system to distinguish infectious non-self (pathogen) from noninfectious self (Janeway and Medzhitov, 2002). To date, 13 TLRs have been identified in mammals and in cattle 10 TLR genes have been mapped. PAMPs specific to viruses are recognized by four TLR family members - TLR3 responds to double-stranded RNA, TLRs 7 and 8 respond to single-stranded RNA, and TLR9 responds to CpG DNA in both viruses and bacteria (Bowie and Haga,2006). TLRs 3, 7, 8, and 9 (viral recognition TLRs, vrTLRs) are candidates for studies of resistance or susceptibility to viral infection in cattle because of their role in a host's defense against viruses.

Foot and Mouth Disease is a leading cause of loss of livestock economy in India. Outbreaks are still being reported from time to time round the year. FMDV during its genome translation and replication undergoes a 'replicative' partially double stranded RNA intermediary form (Belsham,2005) Therefore, in the present study, TLR3 was considered as a candidate molecule for studying certain molecular aspects, since the FMDV RNA undergoes the double stranded "replicative form". It has been assumed that TLR3 is likely to play an important role in host defense against virus infections (Doyle *et al.*, 2003 and Harte *et al.*, 2003) although Zhang et al., (2006) observed that the TLR-3 mRNA expression was not affected by FMDV infection and commented that TLR3 may not be required for the generation of effective antiviral responses to FMDV infection. Moreover, most of the differences in the genome of the individuals of the same species are due to single base substitution polymorphisms, popularly known as single nucleotide polymorphisms (SNPs) and **it was** observed that there was an association between a *TLR3* sequence variant and disease risk in human subjects. However, not much work has been done regarding the role of *TLR3* and its polymorphism in term of SNPs in FMDV infection.

Hence, with the above views in mind, the present work was undertaken to evaluate the expression or detection of certain host factors such as TLR3 (with its SNP analysis) and finally to correlate the observations with severity of the disease.

MATERIALS AND METHODS

Description of samples

In the present study, a total of 52 samples were collected from different parts of Assam and Ranchi which included 40 samples from the FMD affected animals and 12 samples collected as control samples from cattle slaughter houses. The clinical materials comprising of tongue (TE)/feet epithelium (FE) were preserved in 50% glycerol phosphate buffer and were stored in -20^{0}C.

Severity of Disease

The degree of severity of the infected sample was based on the visual observation of the severity of the manifestation of the symptoms. They were graded as 'no disease' for healthy samples and 'less severe' and 'more severe' for diseased samples. On the basis of the severity, out of the total 40 diseased samples, 15 animals were graded as 'more severe' and the rest 25 as 'less severe'.

Serotyping of the samples

In order to confirm the serotype of the samples collected from infected animals , 10 % epithelial suspension were tested by sandwich ELISA as per the bench protocol of Project Directorate on Foot-and-Mouth disease, IVRI campus, Mukteswar, Uttarakhand.

TLR3 Gene Expression by Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction

RNA isolation and cDNA synthesis

Total RNA isolation was done from the tissues using TRIzol (Life Technologies, USA) following the manufacturer's instructions . The isolation was exclusively done using DEPC (Diethoxy pyrocarbonate) treated plastic ware. The total RNA thus isolated was quantitified spectrophotometerically . The quality of total RNA was checked by running 2 µg of the RNA on a 1.8% agarose gel in 1X TBE buffer for 80V for 30 minutes. The RNA was used for cDNA preparation by consulting the methods followed by other workers (Dhara *et. al*.,2007). The cDNA thus prepared was preserved at -20^{o}C.

Semiquantitaive rt-PCR analysis

Semiquantitaive rtPCR was performed for TLR3 using β-actin as internal controls. The primer sequences were designed by using primer3 online software for Exon1of TLR3 (F5'-

TGAGCAGACCTTTGCCTTATC-3'andR5'-GCAGGTGGCAATCTTCTGAG-3') and for ²-actin (F5'-ACTGGGACGACATGGAGAAG-3' and R 5'-GAGGCATACAGGGACAGCAC-3'). The amplicon product sizes for TLR3 and ²-actin were 208bp and 201bp respectively. The PCR conditions were as follows: denaturation at 94°C for 30 sec. annealing at 60°C for 30 sec and extension at 72°C for 45 sec. The PCR buffer contained 10mM Tris HCl (pH 10), 2.0 mM MgCl2 and 50 mM KCl with 0.3U Taq Polymerase (New England Biolab). After 35 cycles, an additional extension at 72°C for 7 min was performed. The amplified product were then subjected to densitometric analysis to compare the band intensity with β actin as the internal control.

Relative Quantification using the Real time PCR

Expression of the RNA transcripts of TLR3 and ²-actin, isolated from the epithelial tissue samples from FMDV infected samples was determined by Real Time PCR (Applied Biosystem) by using SYBR Green Fluorescent Dye. The mRNA levels of the target genes were normalized to the transcript level of the housekeeping gene ²-actin. For relative quantification, the expression of mRNA transcripts of the target genes from normal tissue was also determined. Primers used in this experiment were the same which were used in semiquantitative rtPCR assays as mentioned before. Primers were validated on an Applied Biosystems thermocycler by using serial dilutions of total RNA with endogenous control and target primers, whose values were plotted as the log input amount versus "C_T values (target CT " endogenous CT) for relative efficiency. Primers with a slope of less than 0.1 were used, due to similar amplification efficiencies as the endogenous control.

Beta actin was used as the internal control. The PCR amplification for TLR3 was carried out with initial denaturation at 95°C for 5min; 95°C for 30secs followed by 40cycles 58 °C for 30sec; and a final dissociation step at 72 for 45sec. The readings of the Ct value for the target genes, internal control and calibrator (normal) were obtained and from this, the ΔCt, —dCt and 2^{-dCt} values was calculated. Expression of the target gene normalized to the reference gene and relative to the calibrator = $2^{-\Delta\Delta dCt}$ indicates the fold change in expression of the target gene compared to that in the normal (reference).

Toll Like Receptor 3 (TLR3) Gene Single Nucleotide Polymorphism (SNP) Analysis

Extraction of Genomic DNA

Genomic DNA was isolated from the freshly collected tissue samples for genetic analysis by standard Proteinase-K digestion and phenol/ chloroform extraction procedure (Sambrook et al., 1990).

The quantity and quality of DNA was measured by using Nanodrop spectrophotometer (GE NanoVue plus) following the method as per the manufacturers protocol.

SNP Analysis by PCR Amplification and Sequencing of Exon 3 Region of TLR3 Gene

Primers were designed using primer3 and OLIGO primer designers software (OLIGO version 4.0; National Biosciences Inc., Plymouth, MN) as describe by Dieffenbach *et al*.,(1995) and further consulting the methods given by Chen *et al*., (2003) for amplification of exon 3 region with the sequences reported in the gene bank (accession number: NC 007328) .

The primers were used to amplify a 336 bp product. Briefly, PCR was performed in a total volume of 25 µl containing 50 ng genomic DNA, 200µM dNTPs, 10 pmole of each primer (E4TLR3 **BovF** 5' GCCTTCAGAAGAAC CTCATAACA- 3' and E4TLR3 **BovR** 5' AGCCT TCAAAATGGATGAGC- 3'), 1mM $MgCl_2$ and 0.2U of Taq Polymerase (New England Biolab). Following the initial denaturation step (95°C for 5 min), samples were subjected to 35 cycles of PCR consisting of 94°C for 30 sec, 61°C for 30 sec; and 72°C for 45sec, followed by a final extension for 7 min at 72°C. The amplicon were checked in 2.5% agarose gel prior to direct sequencing of the PCR product at Macrogen® (Seoul, Korea) on commercial basis.

TLR3 Single Nucleotide Polymorphism Analysis

The variations in the nucleotide sequences were verified by comparing with the sequences reported in the National Centre for Biotechnology Information (NCBI) database (gene bank accession number: NC 007328) sequences for polymorphisms and were analyzed using the Clustal X software output (Rosalind Franklin Centre for genomics Research; http://www. hgmp. mcc. ac.uk).The electrophorograms thus generated were

carefully analyzed using the gene runner software as well as manually.

RESULTS AND DISCUSSION

All the 52 samples, comprising of 40 samples from FMDV infected animals and 12 samples from healthy animals, in the form of processed epithelium suspensions (ES) were subjected to serotyping by Sandwich ELISA as per the bench protocol provided by the Project Directorate on FMD, IVRI Campus, Mukteswar, Nainital, Uttarakhand, India. Details of the sandwich ELISA result are given in the Table 1. Out of the total 40 samples from FMDV infected animals, subjected to sandwich ELISA, 29(72.5%) epithelium suspension (ES) were found to be positive for serotype 'O' ,6 (15%) were found positive for serotype Asia1 and 5(12.5%) samples were found positive for serotype A. The samples (N=12) that were collected from slaughter houses as healthy control samples were all found to be negative for any FMDV serotype.

The corrected OD value of the ELISA positive samples ranged from 0.212 to 1.168 compared to the positive control well 2.395 and back ground well 0.01.

Table 1. Detection of FMDV serotype in clinical materials by sandwich elisa

Nature of Samples	No. of Samples tested	No. of samples positive for FMDV serotype			
		Serotype O	Serotype Asia1	Serotype C	Serotype A
Infected Tissue material (ES)	40	29 (72.5%)	6 (15%)	-	5 (12.5%)
Samples from normal animals	12	-	-	-	-

Total RNA isolation was done from the FMDV infected and healthy tissues using the standard TRIzol method which was used for cDNA preparation and amplification of TLR3 Exon1 and β-actin genes with gene specific primers.

Semiquantitative RT-PCR was performed for TLR3 using specific primers and 1µl of cDNA prepared was used as template. β-actin was used as internal controls. An aliquot of 5 µl of the PCR amplified product for each of the genes was run on 2.5% agarose gel in 1X TBE buffer at 80V for 30 minutes The expression of TLR3 at the transcriptional level determined by semi-quantitative RT-PCR was found to be up regulated in FMDV infected cattle compared with controls. (Figure 1)

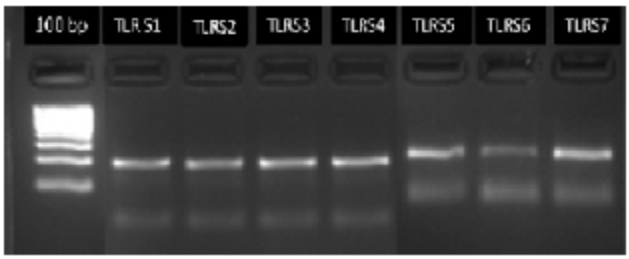


Fig. 1. Representative gel photograph showing PCR amplification of TLR3 amplicons in FMDV infected cattle. Standard 100bp (Fermentas) ladder was used (S1, S2, S3, S4, S7 = infected tissue samples, S5, S6= healthy tissue sample)

Relative quantification study of TLR3 gene of FMDV Infected cattle and healthy control by Real time-PCR

Relative quantification study of TLR3 using β-actin as internal control by real time PCR was performed for validating the observations of relative quantification of TLR3 by semiquantitative RT- PCR. It was revealed that the expression of TLR3 was up regulated in FMDV infected cattle compared to controls. The cycle threshold (Ct) values of the TLR3 transcripts in the samples were obtained from the Real Time PCR analysis (Fig 2).

From the Ct values, ΔCt, —ΔCt and $2^{-\Delta\Delta Ct}$ was calculated and the fold change in TLR3 was interpreted from value of $2^{-\Delta\Delta Ct}$.

The $2^{-\Delta\Delta Ct}$ values of all the samples is depicted in Fig 3 and TLR3 has been found to be upregulated depending on the severity of the diseases from 1.08 fold to 92.41 fold.

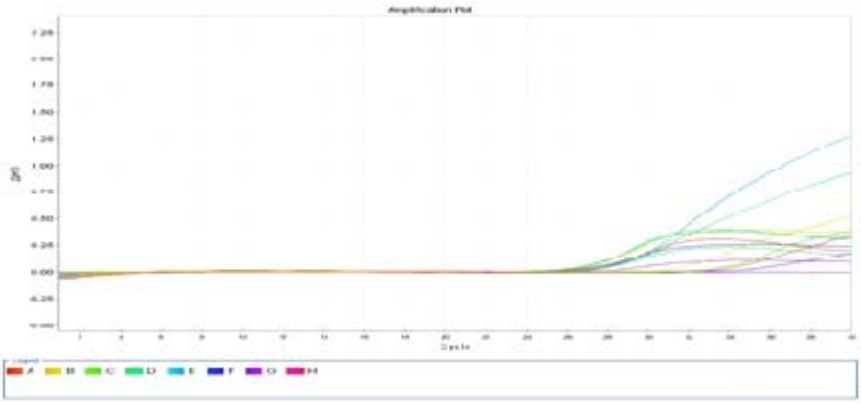


Fig. 2. Linear amplification Real Time-PCR plot showing Ct curve/value of TLR3 and β-actin

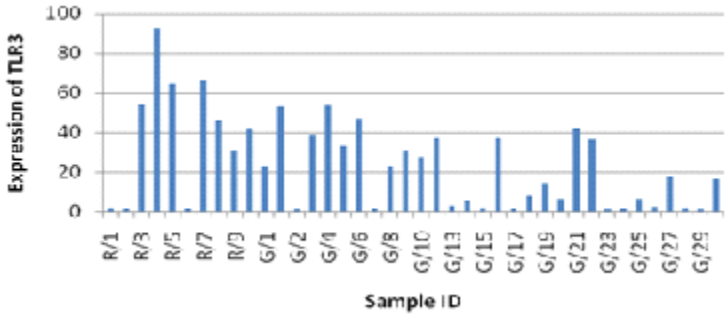
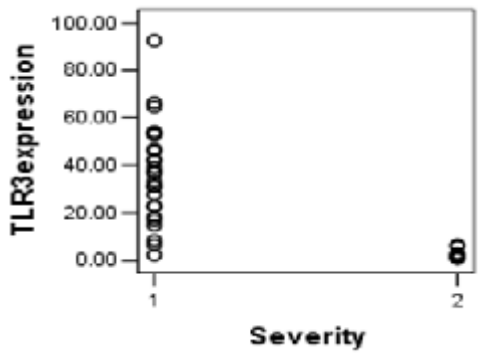
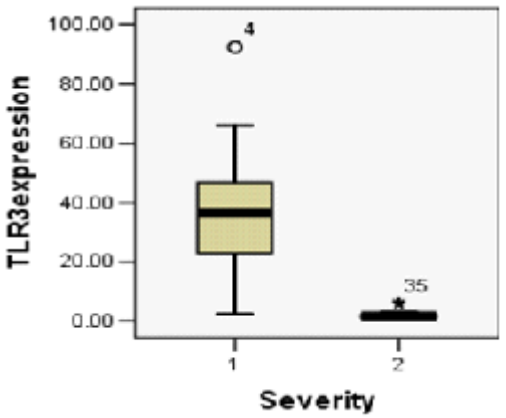


Fig. 3. Bar Diagram showing the fold increase in TLR3 expression in FMDV infected animals



(group 1=less severe case and Group 2=more severe case)

Fig. 4. Distribution of TLR3 expression results in each individual cases of different severity grade



(Group 1=less severe case and Group 2= more severe case)

Fig. 5. Box Plot Analysis showing the mean difference and standard deviation of TLR3 in different groups of cases with different severity grade

Statistical Analysis was carried out for the fold change in TLR3 in FMDV infected cases using SPSSv13 software. Box Plot analysis was done for TLR3 expression against 'less severe' and 'more severe' samples (Figure 4 & 5).

The average expression of TLR3 was found to be 2.187 for more severe compared to 36.321 for less severe cases (Figure 5), the difference of expression of TLR3 being statistically highly significant ($p < 0.001$).

Single Nucleotide Polymorphism (SNPS) of TLR3 Gene Analysis

Genomic DNA was isolated from all the tissue samples from infected (N=40) and healthy (N=12) cases for genetic analysis by standard Proteinase-K digestion and phenol/ chloroform extraction procedure following the method of Sambrook *et al*., 1990. The quantity and quality of DNA was measured by reading the absorbance of the isolated samples at 260 nm (ratio at 260/280 ≤ 1.8) using Nanodrop specrtophometer (GE NanoVue plus) .The fraction showing high purity of DNA was further used for PCR amplification.

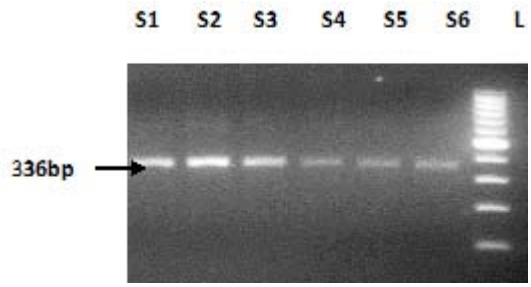


Fig. 6. Representative agarose gel photograph of bovine TLR3 gene amplicon. Standard 100bp (Fermentas) ladder (L) was used (S1, S2, S3, S4, S5, S6 = infected tissue samples)

The TLR 3 gene could be amplified in all the 52 samples. The 336 bp amplicon were checked in 2.5% agarose gel prior to direct sequencing of the PCR product. (Fig.6)

Nucleotide Sequencing of TLR3

All the samples were selected for nucleotide sequencing of the PCR products at Macrogen Inc, Korea. The Exon 3 polymorphism of TLR3 were analyzed by comparing with the sequences reported in the National Centre for Biotechnology Information (NCBI) database (gene bank accession number: NC 007328) sequences for polymorphisms and were analyzed using the Clustal X software output (Rosalind Franklin Centre for genomics Research; http://www. hgmp. mcc. ac.uk).

Analysis Of The Exon 3 Polymorphism Of TLR3 (SNP) With FMDV Infection

TLR3 polymorphism at rs42852440 (-2085 G>A) and rs42852439 (-1991 G>T) were screened in the present study. The analysis were performed by PCR-direct sequencing method with both forward and reverse primer. SNPs rs42852439 mutant allele (either carrier G/T or homozygous mutant T/T) were mostly prevalent in FMDV cases. SNPs rs42852440 mutant allele (either carrier G/A or homozygous mutant A/A) was also mostly prevalent in FMDV cases A few representative partial chromatograms representing TLR3 polymorphism at rs42852440 (-2085 G>A) and rs42852439 (-1991 G>T) have been recorded below (Figure 7, 8, 9 , 10, 11 and 12).

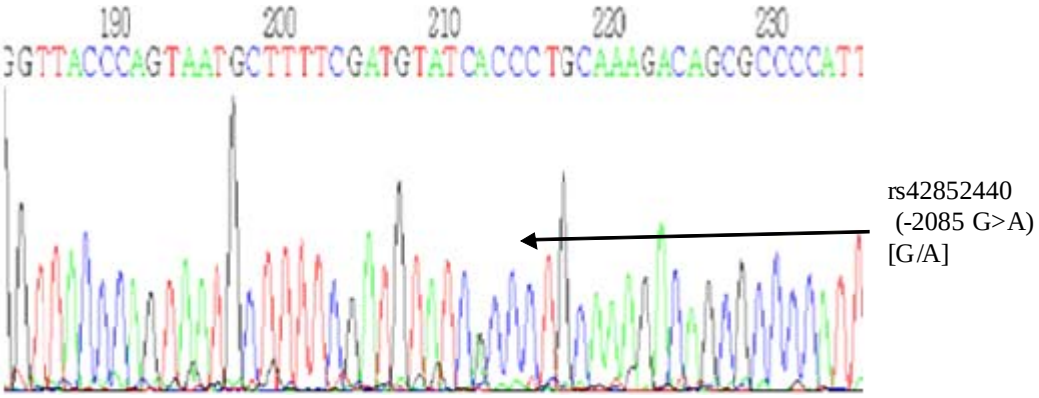


Fig. 7. Partial chromatograms representing Heterozygous [G/A] at position rs42852440 (-2085 G>A) variation

Correlation of Exon 3 SNP of TLR3 with FMDV Infection

In FMDV infected cases, SNPs rs42852439 (-1991 G>T) referred to as GT1 was observed in 60% of the infected cases (24/40) compared to 25% in controls (3/12). The presence of GT1 mutation significantly increased (p=0.030 by Mann Whitney U test) the risk of FMDV infection by more than five folds {Odds Ratio=5.526(1.072-28.492) at 95% C.I, p=0.046(chi square test). Similarly the presence of rs42852440 (- 2085G>A) polymorphism referred to as GT2 was predominant in cases (52.5%, 21/40) compared to controls (16.66%, 2/12), the difference being

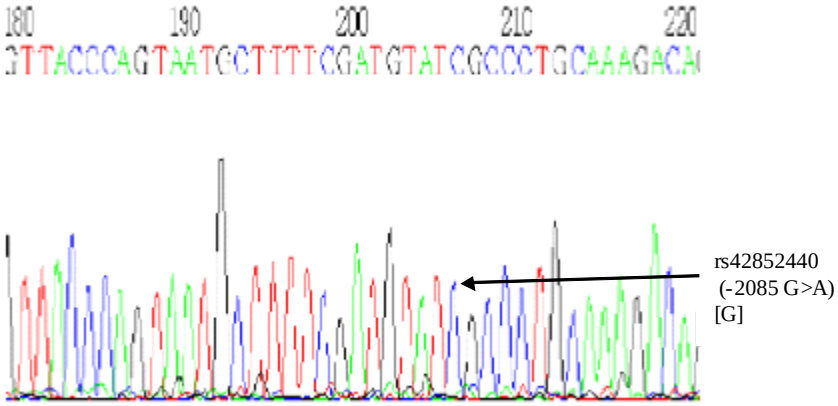


Fig. 8. Partial chromatograms representing Homozygous wild type [G] at position rs42852440 (-2085 G>A) variation

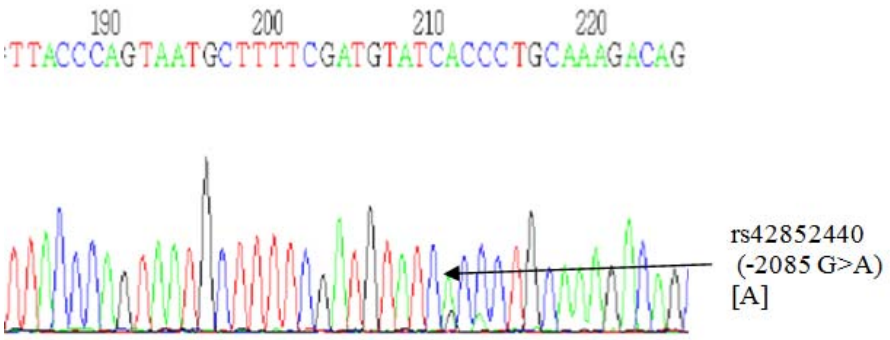


Fig. 9. Partial chromatograms representing Homozygous mutant type [A] at position rs42852440 (-2085 G>A) variation

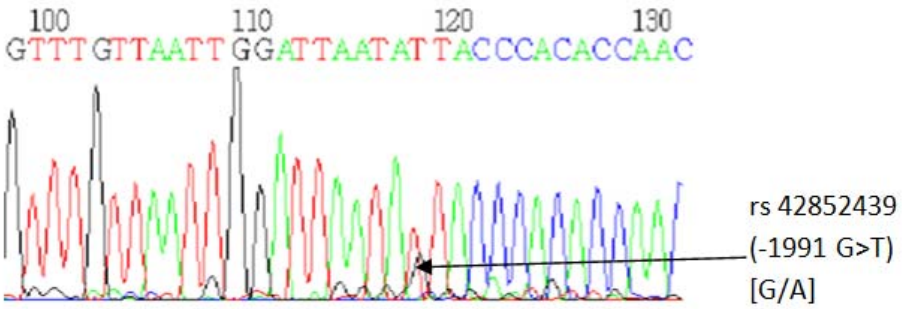


Fig. 10. Partial chromatograms representing Heterozygous [G/T] at at position rs42852439 (-1991 G>T) variation

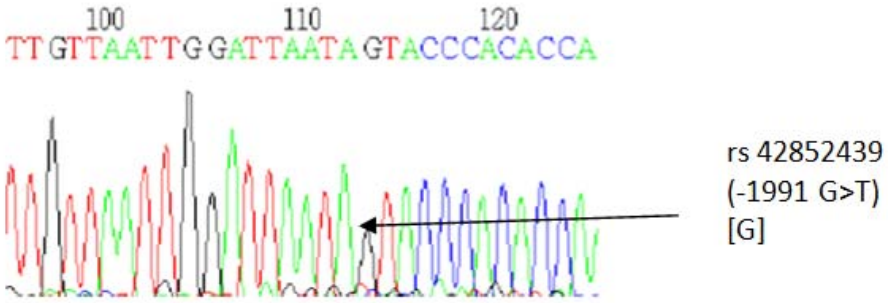


Fig. 11. Partial chromatograms representing Homozygous wild type at position rs42852439 (-1991 G>T) variation

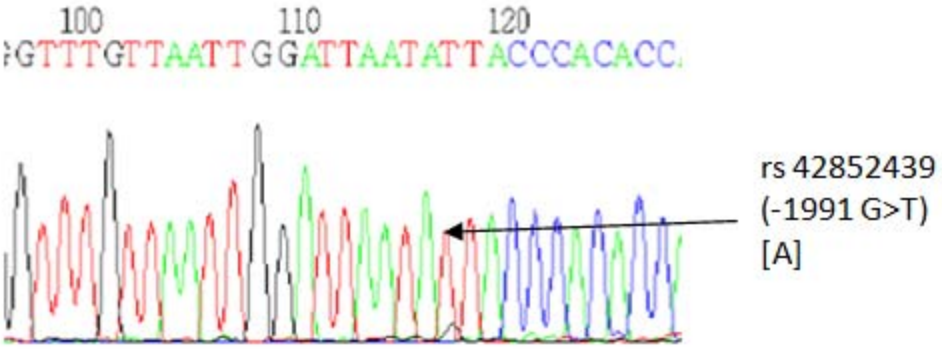


Fig. 12. Partial chromatograms representing Homozygous mutant type [T] at position rs42852439 (-1991 G>T) variation

statistically non-significant (p=0.108). However, the presence of GT2 polymorphism increased the risk of FMDV infection by three folds {Odds Ratio =3.00 (0.773-11.650) at 95% C.I, p =0.186(chi square test)}.

Using the Mann Whitney U test, it was found that, the difference in GT1 (p =0.305) and GT2 (p =0.094) genotype between less severe and more severe groups was not found to be statistically significant. The distribution of neither GT1 mutation {Odds Ratio =0.444 (0.120-1.642) at 95% C.I,p =0.328} nor GT2 mutation {Odds Ratio =0.259 (0.067-1.003) at 95% C.I,p =0.094} tend to influence the severity grade of the disease.

The association of dependence or mutual presence of mutation in GT1 and GT2 cases was found to be statistically non significant (p = 0.275) by Wilcoxon Signed Ranks Test when all the cases and controls for the genotype of GT1(-1991G>T) and GT2 (- 2085G>A) are considered.

Using the Wilcoxon Signed Ranks Test, it was found that statistical correlation was not found between the prevalence or distribution of GT1 genotypes with GT2 genotypes (p=0.439).

Recently many new ''immune-sensing'' receptors for sensing RNA viruses have been identified, including the helicase and TLR families (Kawai and Akira, 2006). For cells of the immune system – in particular DC – TLR3 and TLR7/8 are likely to play important roles. Both represent endosomal sensors of RNA, with TLR3 being triggered by double-stranded RNA while TLR7/8 recognizing single-stranded RNA (Kawai and Akira, 2006).

The investigation of TLR expression in specific cell types, have demonstrated that at least one TLR is present in all tissues examined, and thus, TLRs are ubiquitously expressed. However, differences in the expression of individual TLRs have been observed and seem to depend upon cell types and subsets and activation state of the cell (Muzio *et al.*, 2000 and Hopkins and Sriskandan 2005). Because of their role in a host's defense against viruses, TLRs 3, 7, 8, and 9 (viral recognition TLRs, vrTLRs) are candidates for studies of resistance or susceptibility to viral

infection in cattle. Role of TLR in initial immune responses in human has been the subject of recent studies in various laboratories worldwide. Yet many aspects of molecular events such as their structural binding sites etc. has not been fully elucidated although studies combining different experimental techniques may change this in the future (Kubarenko *et al.,* 2007). Not many literatures are available in the public domain about TLR with respect to FMDV infection in cattle.

In the present study effort was made to correlate expression and mutation in terms of SNPs of TLR3 as well as the association of TLR3 with in disease severity. The severity of the disease was graded as 'more severe', 'less severe' or 'no disease'. TLR3 was found to increase significantly in case of FMDV infected cattle. Out of the total 40 infected samples subjected for TLR3 expression, 14 samples showed marginal expression of TLR3. Among the others, the fold increase in expression varied from moderate to high level. As a whole, the fold increase in the expression of TLR3 varied from 1.08 fold to 92.41. In 'less severe' cases of the disease, TLR3 expression was found to be more and was statistically significant compared to 'more severe' group. This finding was in contrast with the observations made by Zhang et al (2006) while studying on cytokine and TLR mRNAs in the nasal-associated lymphoid tissues in cattle during acute phase of FMDV infection, observed that TLR3 mRNA expression was not affected by FMDV infection, which may indicate that TLR3 may be not required for the generation of effective antiviral responses to FMDV infection. They also recorded that the carrier and non-carrier cattle did not differ in respect of expression of TLR-3 and -4 mRNA in NALT. The issue of organ specific expression of TLRs cannot be ruled out. It has been assumed that TLR3 is likely to play an important role in host defense against virus infections (Doyle *et al.,* 2003 and Harte *et al.,* 2003). Therefore, in contrary to Zhang et al.'s (2006) observation, in the present study, the higher levels of expression of TLR3 in less severe FMD cases indicated that TLR3 might have a role to play in protection against FMDV infection.

When any two human genomes are compared side by side, they are 99.9% identical (Cooper *et al.,* 1985). Most of the differences are due to single base substitution polymorphisms, popularly known as single nucleotide polymorphisms (SNPs). While the majority of the SNPs are of no biological consequence, a fraction of the substitutions have functional significance and are the basis for the diversity found among humans (Collins *et al.,* 1997). In the present study two SNPs (rs42852440 and rs42852439) were targeted within the bovine TLR3 gene. SNPs rs42852439 (-1991 G>T) mutant allele (either carrier or homozygous mutant) were mostly prevalent in FMDV cases. SNPs rs42852440 (-2085 G>A) mutant allele (either carrier or homozygous mutant) was also mostly prevalent in FMDV cases. The SNPs were subjected for correlation analysis with the severity of the disease. The prevalence of G>T was found to be higher in cases compared to controls and the difference in the prevalence of mutated genotypes was statistically significant ($p = 0.030$) but the distribution in the prevalence of G>A mutation was not statistically significant ($p = 0.108$). However, the risk estimate via Odd Ratio analysis indicated that both type of mutations (G>T and G>A) are associated with higher risk of disease. The Wilcoxon Signed Ranks Test analysis for all cases and controls suggested that there was no association of dependence or mutual presence in case of the two SNPs.

CONCLUSION

Recently many new ''immune-sensing'' receptors for sensing RNA viruses have been identified, including the helicase and TLR families with TLR3 being triggered by double-stranded RNA. Therefore, in the present study, TLR3 was considered as a candidate molecule for studying certain molecular aspects, since the FMDV RNA undergoes a double stranded "replicative form". Effort was made to correlate expression and mutation in terms of SNPs of TLR3 as well as the association of TLR3 in disease severity. The fold increase in the expression of TLR3 varied from 1.08 fold to 92.41. In 'less severe' cases of the disease, TLR3 expression was found to be more and was statistically significant compared to 'more severe' group.

The expression of TLR3 was correlated with the disease in that in the 'less severe' cases, TLR3 expression was found to be significantly high compared to 'more severe' cases. The observations

of higher expression of TLR3 in less severe cases suggest the proper operation of the TLR signaling pathway.

The presence of SNPs in the coding region was found to exist although there was no statistical correlation with the severity of the disease.

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