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Identification of SNPs in exon 1, 2 and 14 of ITGβ6 and its association with FMD infection in crossbred cattle of Kerala

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Abstract
The current study involves the analysis of the exons 1, 2 and 14 of bovine ITGβ6 gene and identifies SNPs in association with FMD infection. DNA was extracted from the blood samples taken from crossbred cattle from FMD outbreak areas. PCR-SSCP was performed to identify SNPs in exon 1, 2 and 14. A non-synonymous SNP c.29G>A was identified in exon 2 while a synonymous SNP, c.133C>T was observed in exon 14. Statistical analysis revealed significant association of SNPs with FMD infection ($p \leq 0.01$) in crossbred cattle. The study indicates the potential role of ITGβ6 in FMD infection and hence offers a chance to explore the possibility of breeding for FMD resistance in future.

Keywords: ITGβ6, crossbred cattle, FMD, PCR-SSCP, SNP

Introduction
Foot and mouth disease (FMD) of livestock is a highly contagious viral disease often imparting significant economic setbacks in the dairy industry. The disease could affect cattle, swine, sheep, goats and other cloven-hoofed animals, bringing in production losses mostly in dairy and pig industries with associated heavy mortality in young animals (Kitching *et al.*, 2007) [5]. The Foot and Mouth Disease Virus (FMDV), an *Aphtho* virus of the family *Picornaviridae* occurs in seven immunologically distinct types as strains A, O, C, SAT1, SAT2, SAT3 and Asia1 (Grubman and Baxt, 2004) [4]. A variety of genotypes exist within each of these strains requiring a specific vaccine effective against the circulating viral field strain in the event of an outbreak, to ensure protection. The FMDV binds to specific cell receptors like integrin receptors/heparin sulphate receptors and gains entry into host cells by receptor-mediated endocytosis (Li *et al.*, 2021) [6]. The virus internalization results in *ITGβ6*-FMDV complex, which increases susceptibility to FMD infection (DiCara *et al.*, 2008) [2]. *ITGβ6* facilitates as a receptor for virus attachment. Among different integrin subgroups, *ITGβ6* has the most relevant interaction by acting as a cell surface molecule within the animal host (Lawrence *et al.*, 2013) [6].

Materials and Methods
Collection of samples, DNA extraction
A total of 150 crossbred cattle were selected for the present study. The blood samples were collected from the crossbred cattle maintained at Livestock Research Station (LRS), Thiruvizhamkunnu and farmer's herds at Ayyampuzha. Among these 150 animals, 50 animals were FMDV infected and 100 FMDV non-infected crossbred cattle. Blood samples (4 ml) were collected from the jugular vein into ethylene diamine tetra acetic acid (EDTA) coated vacutainers under aseptic conditions. The samples were transported under the cold chain to the molecular biology laboratory of CASAGB, Mannuthy and stored at -40ºC until DNA isolation. DNA was isolated using Phenol-chloroform method. The concentration, purity and quality of DNA were checked by Nanodrop spectrophotometer (Thermo Scientific, USA). The purity was verified by measuring the absorbance at 260 and 280 nm. The DNA samples with values 1.7 to 1.8 were used for further analysis. The quality of the extracted genomic DNA was assessed by agarose gel (0.8 per cent W/V) electrophoresis. The samples with good quality, purity and integrity were taken for PCR-SSCP analysis.

PCR

Primers for exon 1, 2 and 14 were designed from the bovine *ITGβ6* gene sequence available in the database (GenBank Accession No: ABH04286, whole genome shotgun sequence) using Primer 3 (V.0.4.0) software. The primer sequences are represented in table 1.

Table 1: Sequences of primers designed for PCR

Primer	Sequence (5'-3')	Product size (bp)
EXON 1F	CTACACAAAAATTTCTGCTGAATGA	260
EXON 1R	CACTGTTAAAAGTCTTTCTTGA	
EXON 2F	CAACTTTACACTTCAAGAAAGAAAGA	223
EXON 2R	CAGGTTTGTCAAGCGTACCA	
EXON 14F	CATCTTTTGGAAACTCATTTAACC	230
EXON 14R	TTGTTTTCTCCTTTCCCATGT	

The PCR conditions were optimised in a gradient thermal cycler (Bio-Rad USA) with modifications in different time-temperature combinations of annealing and extension. The PCR mix consisted of 1µl template DNA (50ng/µl), 2µl 10X PCR buffer, 1.2µl $MgCl_2$ (25mM), 0.4µl dNTP's (10mM each), 0.8µl each of forward and reverse primers (10 pM/µl), 0.2µl Taq DNA polymerase (5U/µl) and 13.6µl nuclease free water. PCR was performed in a thermal cycler as per the program described in table 2. The amplicon size was checked by agarose gel electrophoresis (two per cent) along with Gene Ruler 50 bp DNA ladder (Fermentas) as marker. Electrophoresis was performed at 5V/cm for 30 minutes. The gels were visualised under UV light and recorded in a gel documentation system (Bio-Rad, USA).

Table 2: PCR conditions to amplify *ITGβ6* exons

Sl. No.	Step	Temperature and duration		
		Exon 1	Exon 2	Exon 14
1	Initial denaturation	94 ºC (5 min.)	94 ºC (5 min.)	94 ºC (5 min.)
2	Denaturation	94 ºC (30 sec.)	94 ºC (30 sec.)	94 ºC (30 sec.)
3	Annealing	63 ºC (30 sec.)	61.5 ºC (30 sec.)	59.3 ºC (30 sec.)
4	Extension	72 ºC (30 sec.)	72 ºC (30 sec.)	72 ºC (30 sec.)
5	Final extension	72 ºC (3 min.)	72 ºC (3 min.)	72 ºC (3 min.)
(Final hold at 4 ºC) Steps 2 to 4 were repeated for 35 times				

SSCP

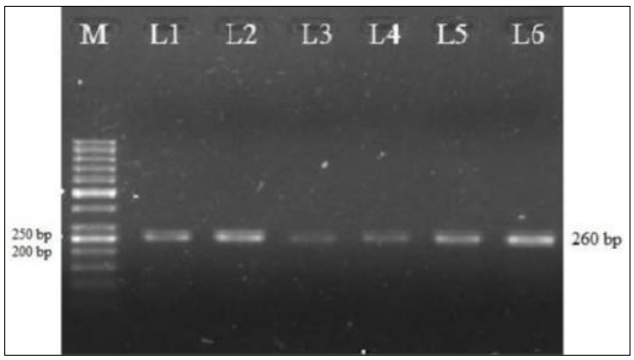
Single strand conformation polymorphism (SSCP) was performed for genotyping of the samples. Six microlitres of amplicons were mixed with 12 µl of denaturing dye, denatured at 95 °C for 10 min and snap chilled immediately on ice. The products were run in 12% polyacrylamide gel (30% acrylamide/bis-acrylamide (29:1) -6ml, 10% ammonium per sulphate - 75µl, TEMED - 15µl, 10X TBE - 1.5ml and nuclease free water – 7.5 ml). Polyacrylamide gel electrophoresis (PAGE) was done at 4°C, for 3.5 h at 120V for exon 1 and 3 h at 100V for exon 2 and 14. Silver staining of the gel was done as recommended by Byun *et al*. (2009) [3] and SSCP fragments were visualised. Representative PCR products showing different banding patterns were sequenced to detect the variations, if any at the nucleotide level. The obtained sequences were subjected to BLASTn to retrieve similar sequences. Statistical analysis was done using SPSS V.24. Chi-square analysis was used for estimating the significance of each genotype in infected and non-infected crossbred cattle. Logistic regression analysis was used for estimating the significance of association of FMD infection with the fixed factors *viz.* age of cattle, location and presence of SNP in the genotype.

Results and Discussion

The concentration and purity of DNA samples were checked using Nano Drop spectrophotometer. The DNA samples with 260/280 ratio between 1.7 and 1.8 were used for the study. The concentration of the DNA was assessed by one per cent agarose gel electrophoresis. Amplification of exons was done using specific set of primers designed using Primer 3 software and procured from Sigma Aldrich. Gel electrophoresis of amplicons confirmed the product lengths. The amplified products of *ITGβ6* exon 1, 2 and 14 are displayed in figure 1, 3 and 6, respectively.

Exon 1

The SSCP analysis of the amplicons of exon 1 of *ITGβ6* in the infected and non-infected cattle revealed only one genotype, AA with two bands for all the samples studied (Figure 2). The same was confirmed by sequencing of representative samples from the infected and non-infected crossbred cattle. The BLASTn analysis discovered only one sequence concerning to *Bos taurus* chromosome 2 which had 100 per cent similarity. The result confirms the conserved nature of the exon 1 in the crossbred population under study.



Lane M: 50 bp DNA marker
Lane 1 to 6: 260 bp product

Fig 1: PCR amplification of *ITGβ6* exon 1

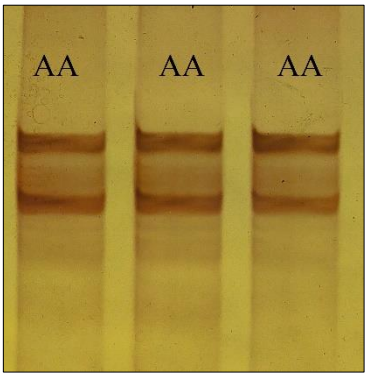
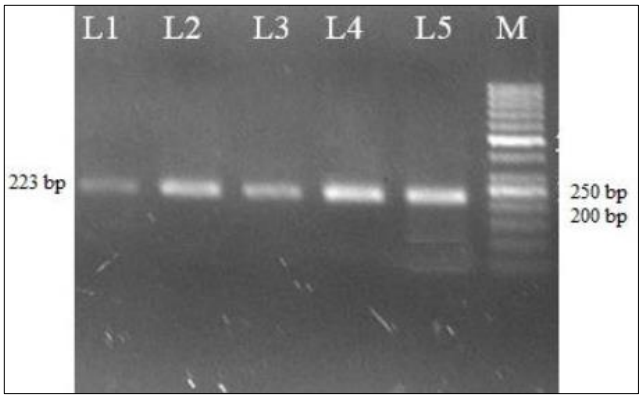


Fig 2: SSCP pattern of *ITGβ6* exon 1 (AA genotype)

Du *et al*. (2009) [3] characterised the Ovine *ITGβ6* in association with FMD in domestic sheep of China. They identified that the exon 1 of Ovine *ITGβ6* had an identical pattern in sheep as well as cattle.

Exon 2

The SSCP analysis of *ITGβ6* exon 2 revealed two genotypes, BB with two bands and BC with four bands (Figure 4). The allele 'B' indicates nucleotide "G" and allele 'C' indicates nucleotide "A". The heterozygote BC (n=91) in exon 2 had a frequency of 0.61 and homozygote, BB (n=59) with a frequency of 0.39. The alleles B and C had frequencies of 0.70 and 0.30, respectively. On sequencing of representative samples from the genotype BC, a non-synonymous SNP at the 29th position of exon 2 was observed (Figure 5). A codon change of AGA to AAA was identified, where AGA being translated to arginine and AAA to lysine. The BLASTn analysis of exon 2 revealed that there was only one similar sequence, the sequence of chromosome 2 in *Bos taurus* which had 99 per cent similarity. Chi-Square analysis revealed a significant difference ($p \leq 0.05$) between genotypes in infected and non-infected crossbred cattle. Logistic regression analysis revealed a significant association ($p \leq 0.01$) of the SNP, c.29G>A in genotype BC and location of the animals with FMD infection while no significant association was found for the age of the animals with FMD infection.



Lane 1 to 5: 223 bp product
Lane M: 50 bp DNA marker

Fig 3: PCR amplification of *ITGβ6* exon 2

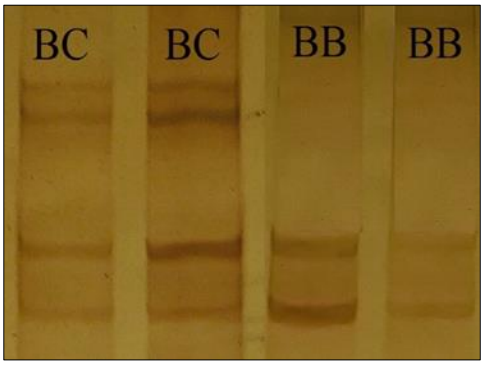


Fig 4: SSCP pattern of *ITGβ6* exon 2 (BB and BC genotype)

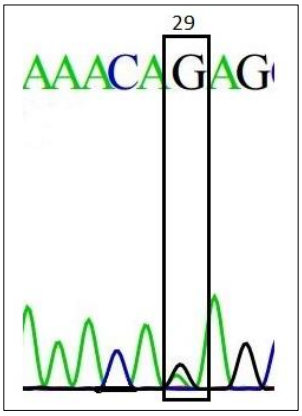
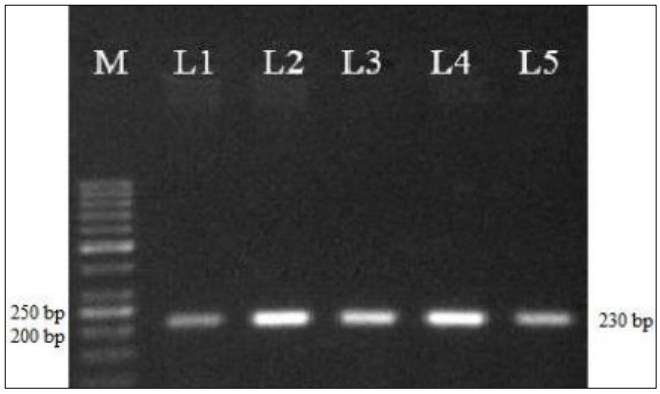


Fig 5: Sequence map of exon 2 of *ITGβ6* showing G→A transition

Singh *et al*. (2014a) [8] performed SNP genotyping using ARMS-PCR in crossbred and indigenous cattle and confirmed the presence of a homozygous genotype (GG) and a heterozygous genotype (GA). The SNP, c.29G>A identified in the present study is in agreement with the SNP reported by Singh *et al*. (2014b) [9].

Exon 14

The SSCP analysis of amplicons of *ITGβ6* exon 14 in the infected and non-infected crossbred cattle revealed two genotypes, II with two bands and HI with three bands (Figure 7). The allele H represent nucleotide 'T' and I represents 'C'. The frequency of the genotype HI (n=82) and II (n=68) of exon 14 in crossbred cattle were 0.54 and 0.46, respectively while allele frequencies for H and I were 0.27, 0.73, respectively. The sequencing of representative samples from the genotype HI, revealed a synonymous SNP at the 133rd position of exon 14 (Figure 8) with a codon change from CTC to CTT, both being translated to leucine. The BLASTn analysis of exon 14 revealed that there was only a similar sequence of chromosome 2 in *Bos taurus* which had 99 per cent similarity. Chi-Square analysis revealed a significant difference ($p \leq 0.05$) between the genotypes in infected and non-infected crossbred cattle. Logistic regression analysis revealed a significant association ($p \leq 0.01$) of the SNP, c.133C>T in genotype HI and location of the animal with FMD infection and there was no significant association for the age of the animal with FMD infection.



Lane M: 50 bp DNA marker
Lane 1 to 5: 230 bp product

Fig 6: PCR amplification of *ITGβ6* exon 14

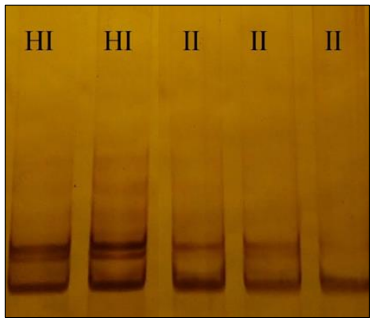


Fig 7: SSCP pattern of *ITGβ6* exon 14 (II and HI genotype)

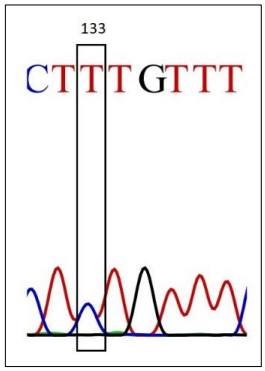


Fig 8: Sequence map of exon 14 of *ITGβ6* showing C→T transition

Singh *et al.* (2015a) [10] studied *ITGβ6* receptor gene in Zebu and crossbred cattle using ARMS-PCR based genotyping. They identified the presence of genotypes 'TT', 'TC' and 'CC' in infected and non-infected Zebu and crossbred cattle. They reported the presence of a similar SNP, c.133C>T in exon 14 of *ITGβ6* in Zebu cattle breeds. Singh *et al.* (2015b) [11] also identified three genotypes *viz.* 'TT', 'TC' and 'CC' in Sahiwal, Kankrej, Ongole and Gir cattle using single tube tetraplex PCR. According to the authors among the three genotypes, TT was widely distributed in the cattle population studied except for Sahiwal. The Sahiwal breed showed a higher frequency of heterozygosity, but the result was not statistically significant. They identified the presence of another SNP c.2145T>C at 2145th position of exon 14 in Zebu and crossbred cattle population.

Conclusion

The FMD is a contagious disease causing major economic loss in Kerala. The *ITGβ6* receptor gene has a main role in disease susceptibility of host to FMD infection. The polymorphisms in *ITGβ6* (exon 1, 2 and 14) and their association with FMD infection was studied in crossbred cattle of Kerala. The current research revealed significant association of SNPs within genotypes with FMD infection. The results obtained from the present study indicate the potential role of *ITGβ6* on FMD infection. This opens up the possibility for further exploration of *ITGβ6* gene as one of the potential candidate marker genes for FMD. However larger sample study is required to identify the distribution pattern of the gene and effect of polymorphism on FMD infection/non-infection in crossbred cattle of Kerala.

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