Heterozygosity at the SNP (rs136500299) of ITGB6 receptor gene possibly influences the susceptibility among crossbred bull to foot and mouth disease infection

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Abstract Zebu (*Bos indicus*) cattle are known to be resistant against foot and mouth disease virus (FMDV) compared to taurine (*Bos taurus*). To understand the susceptibility of two cattle species to FMDV infection in terms of viral receptors, the present study reports the cloning, characterization and sequence analysis of Zebu ITGB6 gene. The complete CDS of zebu ITGB6 was 2367 base-pair in length with 788 amino acid residues. The zebu integrin shares common structural and functional elements with taurine and other species. We identified an amino substitution (S₆₆₅ to F₆₆₅) presents in ITGB6 gene among zebu and taurine as SNP (rs136500299). Further, we determined and compared the structural differences of ITGB6 receptor gene among zebu and taurine species. To elucidate the influence of the SNP on the susceptibility of cattle to FMDV infection, a tetra ARMS PCR based genetic screening was performed among Zebu and crossbred cattle. Our observation revealed that, the targeted SNP are strongly ($P < 0.05$) associated with FMD susceptibility among Frieswal (HF X Sahiwal) crossbred cattle.

Keywords ITGB6 · FMDV · SNP · Frieswal

Introduction

Integrins are a group of heterodimeric receptors with α and β subunits, having a single large extracellular domain, two transmembrane domains and a short C terminal cytoplasmic domain [6]. It is well known that many of the integrins can recognize RGD (Arginine–glycine–aspartic acid) motifs displayed on the extracellular matrix (ECM) proteins of viral capsid, which after binding to the integrin may change the conformational structure [15]. It is well established that foot and mouth disease virus (FMDV) can initiate infection in vitro by virtue of its binding capacity to the integrin family cellular receptors (aVb1, aVb3, aVb6 and aVb8) via its highly conserved RGD sequence motif located within its bG–bH loop of VP1 protein [1, 7–9]. Integrin aVb6 (ITGB6) is widely believed to be a high-affinity receptor for the Foot-and-mouth disease (FMD) virus tropism among infected animals [5].

FMD is an important viral disease affecting cloven hoofed animals, mainly cattle, swine, sheep and goat, which causes severe economic losses [16]. The etiological agent of FMD is FMD virus, a member of the genus *Aphthovirus* of the *Picornaviridae* family and is classified into seven distinct serotypes (O, A, C, SAT-1 to 3, and Asia-1) and numerous subtypes [10]. Cattle are more susceptible to aerosol infection than other ruminant species. It is well known that, Zebu (*Bos indicus*) cattle are less susceptible to FMD than taurine (*Bos taurus*) [10]. It is not yet established the genetic basis which contributes to the FMD resistant phenotype for the bovine host. Du et al. [2–4] reported that, host tropism of FMDV may be related to divergence in integrin subunits among the Bacterian and dromedary camel. At present, no retrieval nucleotide sequence of *B. indicus* ITGB6 is available. Therefore, to understand the susceptibility among zebu and taurine cattle

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with respect to ITGB6 gene receptor, the present experiment was designed to clone, characterize and analyze the complete CDS of ITGB6 among Indian Zebu (*B. indicus*) and further to identify divergence in ITGB6 sub-units of this species. The aim of the present investigation was focused to identify genetic polymorphism within a SNP (rs136500299) located at exon 14 region of bovine ITGB6 and their association with FMD susceptibility among crossbred bulls.

Materials and methods

RNA isolation

Tongue tissue samples were collected from the zebu (*Bos indicus*) cattle from a slaughter house, Kerala, India. Approximately 400 – 500 mg of the sample was kept in liquid nitrogen until use. Tissues were grinded thoroughly with an RNase-free, liquid-nitrogen-cooled mortar and pestle. Total RNA from each tissue was extracted with RNeasy Mini Kit (Sigma-aldrich, USA) as described by the manufacturer. The extracted RNA was quantified spectrophotometrically and the OD260/OD280 was used for evaluation of purity. The integrity of the RNA was checked by visualization of 18 s and 28 s ribosomal bands on an agarose gel.

Amplification of cDNA

Reverse transcription of total RNA was carried out using a ProtoScript first strand cDNA synthesis kit (New England Biolabs, Beverly, MA, USA) as per the manufacturer's recommendations employing the M-MuLV reverse transcriptase and random primers. The cDNA product was stored at -20°C . The ITGB6 cDNA was amplified from cDNAs of zebu cattle tongue tissue by PCR using primers based on *B. taurus* integrin sequences available in NCBI GenBank database. A fusion-PCR technique was designed for reconstruction of the *B. indicus* complete ITGB6 gene using four sets of overlapping degenerated primers (Table 1). The strategy for amplifying full length CDS of ITGB6 has been

depicted in Fig. 1a. The resulting PCR products were run on 1 % agarose gel containing ethidium bromide and the DNA bands were visualized using a UV transilluminator.

Cloning and sequencing

Each individual amplified products corresponding to integrin cDNAs from tongue tissue samples were gel-purified using the Gel Extraction Kit (Qiagen, Germany). The purified products were ligated into the pTZ57/R vector (InsTAclone PCR Cloning kit, Fermentas) and the resultant recombinant plasmids were transformed into competent *Escherichia coli* strain DH5 alpha. Positive clones were selected on ampicillin/IPTG/X-Gal plates and plasmid DNA was isolated and purified with Plasmid Miniprep Kit (Qiagen, Germany) by the manufacturer's protocol. Representative plasmid of each clones subjected for sequencing.

Sequence analysis

Primer design was performed with oligo6.0 software. Sequence data analyses were performed using the BLAST search of the NCBI. The sequence homology; alignment and divergence were calculated using the Laser-gene analysis software package (DNASTAR, USA). The nucleotide and amino acid sequence were aligned using Clustal W program available in the BioEdit v7.0.5 software package (Ibis therapeutics, Carlsbad, CA). Phylogenetic tree were constructed using MEGA version 3.1. These sequence data herein have been submitted to GenBank and accession number is KF886535. The reference sequences included in the analysis were taken from GenBank accession numbers (DQ867017, EF613220, GQ443501, EF432729, JQ965817, NM001113772, NM001004263, NM000888, XM005601534, XM852055, XM003990799, XM001094740 and XM001149234).

Collection of biological samples from FMD infected animals

During an incidence of FMD, a total thirty eight Frieswal (HF $\times$ Sahiwal) cross bred bulls, maintained at

Table 1 Primer sequence details for ITGB6 gene

S. no.	Primer code	Primer sequence	Position	Amplicon size (bp)
1.	ITGB6 F1	CTGTCCAGGTAGCYTCTGTTT	51–71	690
	ITGB6 R1	TACTGCAAGGGTTRGCGATTT	720–740	
2.	ITGB6 F2	GACGACCTCAACACCATCAA	584–603	714
	ITGB6 R2	CTTCYGTGTCTCCTAACACTTC	1277–1298	
3.	ITGB6 F3	CTGTCACTTGGACAGCAAGA	1021–1040	803
	ITGB6 R3	CAGTCACAGTCGCCRTTATCT	1804–1824	
4.	ITGB6 F4	GGAAACATTTAYGGGCCTTACT	1733–1754	835
	ITGB6 R4	GTGCAWTAACATTTTCATATCAG	2547–2566	

(Y: Degenerate bases)

Fig. 1 Characterization of the complete CDS of *Bos indicus* ITGB6 gene by overlapping primer walking. **a** schematic representation of overlapping primer walking strategy used. **b** PCR amplified product with four sets of primer. Lane 1 600 bp, Lane 2 714 bp, Lane 3 803 bp, Lane 4 830 bp, M 100 bp ladder

institutional Bull Rearing Unit (BRU) were divided into two group according to the FMD gross lesions. r3AB NSP ELISA were conducted for further screening of sero positivity of the serum samples. Animals were maintained under similar feeding and managerial practices. The first group was composed of the bulls that had never been affected by FMD infection kept as the control groups ($n = 19$) according to their history records (NSP ELISA negative). The second group were consist of those bulls whose sera were positive for r3AB NSP ELISA ($n = 19$), designated as case. Blood samples were collected from all the animals by jugular vein puncture using sodium heparin (10 IU/ml) as an anticoagulant. Immediately after collection, blood samples were stored in a portable refrigerator at 4 °C, transported to the laboratory, and stored at -80 °C until DNA extraction. The genomic DNA was extracted

from venous blood using phenol–chloroform extraction method [13] and the purity of genomic DNA was assessed spectrophotometrically.

Genotyping

Tetra ARMS PCR primer designing

The tetra-primer PCR procedure [17] was used for genotyping the SNP (rs136500299) mutation in the 14th exonic region of ITGB6 receptor gene. The method employs four primers to amplify a fragment from DNA containing the SNP and amplicons representing each of the two allelic forms. Primers can be designed to amplify fragments of differing sizes for each allele band in order for them to be easily resolved using agarose gel electrophoresis. Primers

Table 2 Primer designed for tetra ARMS PCR based genotyping

Tetra primer codes	Primer sequence (5'–3')	Amplicon size
Outer forward	TGCATAATAAAACTCAATAC	TT: 433 and 347 bp
Outer reverse	ATTCATCAGCCACCTTTTTC	TC: 433, 347 and 128 bp
Inner forward	CAGATTTCTCAAAGGATAGCTT	CC: 433 and 128 bp
Inner reverse	CTTGCGAGAGAACAGGAAACAG	

(Bold letter indicates mismatch)

Fig. 2 a Analysis of deduced nucleotide sequence variation at *Bos indicus* ITGB6 gene revealed a Missense variant at 2145 position in the transcript [T2145C, TTT: Phenylalanine to TCT: Serine]. **b** Dendrogram representing the missense mutation

were designed according to bovine ITGB6 sequence. 'BLAST' program (<http://www.ncbi.nlm.nih.gov/blast>) was used to check the specificity of the primers. Details of the primer used for the present study and the amplicon size for different genotypes shown in Table 2.

Polymerase chain reaction and sequencing

PCR was performed in a total volume of 25 µl containing approximately 50 ng DNA, 2.5 µl of 10X buffer, 2.0 mM MgCl₂, 0.2 mM dNTPs, 5 pmol of each outer primers, 10 pmol of each inner primers and 1U of Taq polymerase (Sigma-Aldrich, USA). The polymerase chain reaction (PCR) protocol was 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 10 min. The PCR products were separated on 1.0 % agarose gel (Sigma-Aldrich, USA) including 0.5 µg/ml of ethidium bromide, photographed under Gel Documentation system (Alpha imager® EP). Amplified PCR products were gel purified

(Fig. 4). Further validation of three different genotypes was confirmed by cloning and sequencing of the 433 bp PCR products of each genotypic group.

Statistical analysis

Gene (allele) and genotype frequencies were calculated by direct counting method and Chi square (χ^2) analysis was performed to test the association of the different genotypes with FMD susceptibility in Frieswal bulls.

Results

Sequence analysis and variant identification

Figure 1 represents the overlapping PCR products for the complete CDS of *B. indicus* ITGB6 gene. Figure 1b shown the different amplified fragments of ITGB6 using four sets of primers. Figure 2 showed the deduced nucleotide

Phylogenetic tree showing relationships between various species. The tree is rooted at the bottom left. The species listed on the right are: Bos indicus (marked with a black dot), Bos taurus, Goat, Sheep, Bacterian Camel, One humped Camel, Pig, Horse, Dog, Cat, Monkey, Human, Chimpanze, and Rat. Bootstrap values are shown at the nodes: 99 for the node joining Bos indicus and Bos taurus; 100 for the node joining Goat and Sheep; 99 for the node joining Bacterian Camel and One humped Camel; 55 for the node joining the (Goat, Sheep) clade and the (Bacterian Camel, One humped Camel) clade; 96 for the node joining the (Goat, Sheep, Bacterian Camel, One humped Camel) clade and the Pig; 99 for the node joining Dog and Cat; 100 for the node joining Monkey and Human; 70 for the node joining Human and Chimpanze; and 100 for the node joining the (Monkey, Human, Chimpanze) clade and the Rat.

of them share all common structural and functional elements of the ITGB6 molecule.

The complete coding sequence for the *Bos indicus* ITGB6 was found to be 2367 nucleotides in length encoding 788-amino-acid-residue protein. The correctness of the obtained sequence was confirmed from published WGS data of Zebu. Analysis and alignment of deduced CDS region of the *Bos indicus* ITGB6 (KF886535) with NCBI retrievable three mRNA transcript isoforms (X1, X2 and X3) of Herford cattle breed ITGB6 sequences (XM_005202409.1, XM_005202408.1 and XM_005202407.1 respectively) revealed that, at least one non synonymous/missense variation at 2145th position of transcript (667th at protein) where the amino acid phenylalanine changed to serine (TTT to TCT). Further, validation of the identified SNP was confirmed from the ITGB6 *B. taurus* Ensemble Genome Browser (dbSNP: rs136500299) sequence located at Chromosome 2:36336237. From the scrutiny of deduced amino acid sequence (AH148847) of *B. indicus* ITGB6, it revealed that the protein shares common structural and functional elements with ITGB6 molecules from the other species including *B. taurus*. At the N-terminal end, the protein contains a putative signal peptide of 26 residues (M₁-G₂₆), an ecto/extracellular domain of 681 residues (G₂₇-N₇₀₇), a hydrophobic transmembrane domain of 29 residues (I₇₀₈-F₇₃₆), and a cytoplasmic tail region of 52 residues (H₇₃₇-G₇₈₈). The *Bos indicus* ITGB6 subunit contains 7 possible N-linked glycosylation sites and one of them (NVT) located in cytoplasmic tail portion of ITGB6. The protein possesses 58 cysteine (C) residues, among them majority (31) are located within the cysteine rich domain (C₄₅₆-T₆₁₉). 3, 6, 4, 4, 9 and 1 residues are located within the signal peptide,

Identifying the genetic makeup responsible for mediating naturally acquired FMD resistance among zebu cattle is quite interesting and need to be initiated. In the present investigation we successfully cloned and sequenced the complete ITGB6 receptor gene CDS/transcript of zebu (*B. indicus*) cattle for the first time. Further, our preliminary findings from the structural point of view between zebu and taurine revealed that, despite some focal differences, both

Fig. 4 a Agarose gel electrophoresis of the tetra-primer PCR assay products of identified SNP T 2145 C. *Lane 1* TT genotype (433 and 347 bp), *Lane 2* CC genotype (433 and 128 bp), *Lane 3–4* TC genotype (433, 347 and 128 bp). **b** Dendogram representing the heterozygous genotypes

Table 3 Association of T2145C SNP with FMD infection among crossbred bull

Status	Genotypes			Total	Chi square	p value	TC vs TT		TC vs (TT + CC)	
	TT	TC	CC				OR (CI)	p value	OR (CI)	p value
Case	5	14	0	19	11.11	0.00387	9.10	0.0043	10.5	0.0022
Control	13	4	2	19			(1.998–41.446)		(2.3357–47.2025)	

OR odd ratio, CI 95 % confidence interval

ectodomain 1, ligand binding region, ectodomain 2, ectodomain 3 and transmembrane regions respectively. Most of the cysteine residues are conserved across the species and probably may involved in disulfide bridge formation [3]. The extracellular/ectodomain contains the ligand binding region of 242 amino acids (Y₁₃₁–R₃₇₂). It includes a putative metal-ion-dependent-adhesion-site or MIDAS (residues D₁₄₀, S₁₄₂, S₁₄₄ and E₂₄₀). Cytoplasmic tail contains one conserved “NP_{XY}” motif at positions 759–762. Phylogenetic analysis of deduced protein sequence using neighbor joining method revealed that, ruminants are distinctly place as a separate node and *B. indicus* and *B. taurus* share 99 % similarity.

Recently, Singh et al. [14], reported that SNP at 5'UTR region of crossbred cattle are significantly associated with FMD susceptibility. To identify whether the identified SNP at exonic region are polymorphic among different Zebu breed, we developed a tetra ARMS PCR based genotype detection tool for screening the SNP across different Zebu cattle breed. The tetra ARMS-PCR is a proficient polymorphism detection tool which is based on conventional PCR technique. It is practical for SNP detection in a single

step PCR without the need of downstream processing like RFLP/SSCP [11] or a nested PCR [12].

To shed light on the effect of the SNP (rs136500299) on the FMD susceptibility, we further evaluated the screening of genetic polymorphism among crossbred cattle (Frieswal) after an incidence of FMD. We classified the animals based on their FMD outbreak as case and control. The same tetra ARMS PCR technique was implemented for screening of polymorphism among case and control groups.

When we look for the association of the genotypes with FMD susceptibility, it makes us clear that, the SNP T2145C are strongly ($P < 0.01$) associated with the occurrence of the disease, where the TT genotypic groups are less susceptible to FMD compare to other. Present findings concluded that, T2145C SNP at exonic region of bovine ITGB6 gene may provide some reference to be implemented for FMD disease resistant breeding.

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