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Single tube tetraplex PCR based screening of a SNP at exon 14 region of bovine ITGB6 among different Zebu breeds

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ABSTRACT

The present study was aimed to screen genetic variation within a SNP (rs136500299) located at exon 14 region of bovine ITGB6 gene among different Zebu cattle breeds. The genotyping method describe in the present study is a tetraplex ARMS PCR, which offers extremely fast, economical, and simple detection tool. The distribution of the ITGB6 genotypes among the different breeds studied suggested that the populations were under Hardy–Weinberg equilibrium. Our findings revealed that TT genotypes are widely distributed among different Zebu cattle breeds, which can be associated with the resistance to FMD virus, as the *Bos indicus* are more resistant to FMD virus in comparison to *Bos taurus*.

Introduction

Foot and mouth disease (FMD) is one of the highly contagious diseases of domestic animals, caused by foot-and-mouth disease virus (FMDV), a member of the family Picornaviridae, which has a colossal global impact, due to the huge number of animals affected. The FMD endemic countries collectively contain three-quarters of the world's population (Robinson et al., 2011), indicating the global economic significance of the disease. At the national level in India, annual total economic loss due to FMD ranges from Rs 12,000 crore to Rs 14,000 crore (Singh et al., 2013). In spite of all the control measures taken, FMD continues to be

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an economically important disease in India due to poor surveillance and inadequate control programs. Understanding of the mechanism of Foot-and-Mouth disease virus (FMDV) infection and replication of this virus is important to the control of this worldwide menace (Alexandersen et al., 2003; Longjam et al., 2011). A vital question that has yet to be addressed concerns the role of viral receptors in the pathogenesis of FMD. Several integrins have been identified as FMDV receptors. Integrins are the biologically important set of proteins used by the cells to bind and respond to the extracellular matrix which belong to a large family of integral membrane receptors that is required for cell adhesion. Functionally active integrins consist of two noncovalently bound transmembrane glycoprotein subunits viz, alpha (α) and beta (ß) (Springer, 2002). Evidence suggested that virus binding and infection of the Integrin ß 6 (ITGB6) transfected cells are mediated through an RGD-dependent interaction (Baxt and Becker, 1990; Jackson et al., 2000; Mason et al., 1994). Considering the role of host ITGB6 receptor gene, the aim of the present study was to screen genetic variation within a SNP (rs136500299) at exon 14 region among different indigenous cattle. The polymorphism T/C is located at position 2145 of the reference ITGB6 mRNA and produces a missense change Phe/Ser in the position 667 of the polypeptide. Previously, this polymorphism has been reported at least ten times: ss250661448, ss263658018, ss415630763, ss420604742, ss422472086, ss679762826, ss752601716, ss828200981, ss907724252, and ss942564461. In this study, we describe an accurate method on the basis of competitive polymerase chain reaction (PCR) so called tetraplex ARMS PCR, for identification of the SNP among different animals.

Materials and methodology

For analyzing the scenario of targeted SNP among different Zebu cattle, a total of 148 animals of different indigenous breeds of cattle {Sahiwal (51), Kankrej (48), Ongole (38) and Gir (11)} from different agricultural zones were used in the study. 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 (Sambrook and Russell, 2001) and the purity of genomic DNA was assessed spectrophotometrically. The tetra-primer PCR procedure (Ye et al., 2001) was used for genotyping the SNP (rs136500299) at exon 14 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 were designed according to bovine ITGB6 sequence (http://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi). '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 were shown in Table 1. PCR was performed in a total volume of 25 μl containing approximately 50 ng DNA, 2.5 μl of 10X buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 5 pmol of each outer primers, 10 pmol of each inner primers and 1 U of Taq polymerase (Sigma–Aldrich, USA). The polymerase chain reaction (PCR) protocol was 94 °C for 5 min, followed by

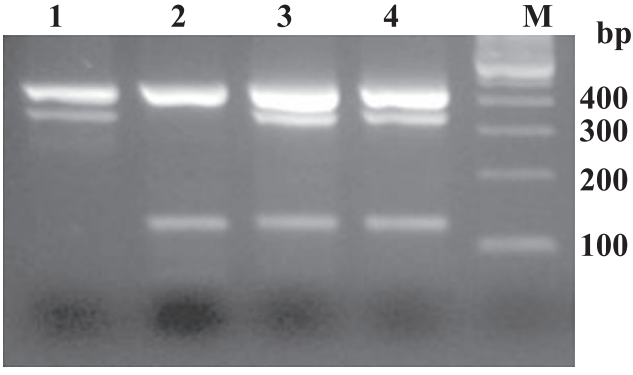

Fig. 1. Agarose gel electrophoresis of the tetra ARMS PCR based assay products of identified SNP T 2145 C. Lane 1: TT genotype (433 and 347 bp), Lane 2: CC genotype (433 and 128 bp), Lane 3 & 4: TC genotype (433, 347 and 128 bp), M: Molecular weight marker.

Table 1
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	ATTCATCAGCCACCTTTTTG	TC: 433, 347 and 128 bp
Inner forward	CAGATTTCTCAAAGGATAGCT**T**	CC: 433 and 128 bp
Inner reverse	CTTGCAGAGAACAGGAAACA**G**	

(Bold letter indicates mismatch).

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 and photographed under Gel Documentation system (Alpha imager® EP). Amplified PCR products were gel purified. Validation of the three different genotypes was analyzed by cloning and sequencing the 433 bp PCR products for each genotype. Gene (allele) and genotype frequencies of ITGB6 receptor gene were calculated by direct counting method. The four populations were tested for Hardy–Weinberg equilibrium using PROC ALLELE (SAS Inst. Inc., Cary, NC). The Chi-square (χ^2) test was used to find the difference in genotype frequencies in different breeds.

Results and discussion

The present study was aimed to develop a single tube tetraplex PCR based genotyping assay for SNP (rs13650029) at exonic region of bovine ITGB6 receptor gene. SNP genotyping techniques depend on amplification of the target DNA using PCR technique, but differ in the means of discerning between the different alleles, which involves significant post-amplification manipulations. For instance, the restriction fragment length polymorphism typing method based on digestion of amplified PCR products with suitable restriction endonuclease. Another extensively used SNP typing technique allele specific oligonucleotide (ASO) melting involves lengthy blotting and hybridization techniques. Tetra-primer ARMS–PCR method described in the present context circumvents the limitations of the earlier mentioned techniques. The technique used in the present study involves a single step PCR protocol with two sets of primers to detect the different banding pattern, without the downstream processing like RE digestion or hybridization. The primers were designed in such a way to amplify fragments of differing sizes for each allele, in order to resolve differentially in agarose gel electrophoresis. The method described in the present study is a simple, swift and cost-effective method for SNP genotyping in large number of individuals (Ye et al., 2001). Correspondence between ARMS PCR and sequencing confirmed three genotype pattern of the targeted SNP at exon 14 region of bovine ITGB6 gene among different Zebu cattle breeds (Fig. 1). Genotyping revealed that, the genotype TT is widely

Table 2
Chi-square test for Hardy–Weinberg equilibrium with respect to the ITGB6 receptor gene in different indigenous breeds of cattle.

Breed	Gene frequencies		Observed genotype frequencies			Expected genotype frequencies			Chi-square (χ^2) value	P-value
	T	C	TT	TC	CC	TT	TC	CC		
Sahiwal (51)	0.750	0.250	28	20	3	28.688	19.125	3.187	0.068	0.9668^{NS}
Kankrej (43)	0.850	0.150	32	9	2	31.068	10.965	0.967	1.484	0.4763^{NS}
Ongole (37)	0.838	0.162	26	10	1	25.973	10.054	0.973	0.001	0.9995^{NS}
Gir (11)	0.955	0.045	10	1	0	10.023	0.955	0.022	0.024	0.9880^{NS}
Total			96	40	6	95.752	41.099	5.149	142	

Numbers in parentheses indicate the number of animals in each breed.
^{NS} Non-significant.

Table 3
Chi-square test for the genotypic frequency of ITGB6 receptor gene among different indigenous breeds of cattle.

Breed	Observed genotype frequencies			Expected genotype frequencies			Chi-square value	P value
	TT	TC	CC	TT	TC	CC		
Sahiwal (51)	28	20	3	34.48	14.37	2.15	7.900 at 6 df	0.2455[NS]
Kankrej (43)	32	9	2	29.07	12.11	1.82		
Ongole (37)	26	10	1	25.02	10.42	1.56		
Gir (11)	10	1	0	7.43	3.10	0.47		
Total	96	40	6	96	40	6		

[NS] Non-significant.

distributed among the targeted Zebu cattle breeds. The study revealed that the frequency of T allele was higher in indigenous populations compared to the C allele. The T allele was mostly observed in the homozygote condition than in heterozygotic individuals. This trend was mostly seen in all the breeds except Sahiwal where the heterozygotic frequency was comparatively higher, even though the difference is not statistically significant (Table 2). The test for HW equilibrium among different populations showed that all the breeds were under equilibrium with respect to the ITGB6 receptor gene indicating the absence of aggressive selection i.e. selection primarily based on this gene ($P > 0.05$). The frequency of animals having CC genotype was lowest in all the four breeds. In order to study the variation in the genotype frequencies among different indigenous breeds of cattle, Chi-square test was performed. The result showed in Table 3 indicates that the genotype frequencies were not statistically different ($\chi^2 = 7.900$, $P = 0.2455$) among the breeds indicating that all the populations are having similar genetic constitution with regard to the ITGB6 receptor gene. The SNP (rs136500299) T/C located at position 2145 of the reference ITGB6 mRNA produces a missense change Phe/Ser. Serine is a polar amino acid with its smaller size, whereas phenylalanine is an aromatic amino acid with complex structure. Similar kind of observation was earlier made by Pan et al. (2008). Thus it may be presumed that changes of amino acid may alter the conformational changes of the ITGB6 receptor coding polypeptide, which however, needs to be confirmed through further studies. The results of the present study suggest that T allele is widely distributed among the indigenous breeds of cattle which can be associated with the resistance to FMD virus, as the susceptibility to FMD virus is lower than the tauras breeds. However, studies with larger sample sets and wide range of cattle breeds are still needed to confirm the exact genetic distribution pattern of the SNP.

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References

Alexandersen, S., Zhang, Z., Donaldson, A.I., Garland, A.J., 2003. The pathogenesis and diagnosis of foot-and-mouth disease. J. Comp. Pathol. 129, 1–36.

Baxt, B., Becker, Y., 1990. The effect of peptides containing the arginine–glycine–aspartic acid sequence on the adsorption of foot-and-mouth disease virus to tissue culture cells. Virus Genes 4, 73–83.

Jackson, T., Sheppard, D., Denyer, M., Blakemore, W.E., King, A.M.Q., 2000. The epithelial integrin αvß6 is a receptor for foot-and-mouth disease virus. J. Virol. 74, 4949–4956.

Longjam, N., Deb, R., Sarmah, A.K., Tayo, T., Awachat, V.B., Saxena, V.K., 2011. A brief review on diagnosis of Foot-and-Mouth Disease of livestock: conventional to molecular tools. Vet. Med. Int. 905768 http://dx.doi.org/10.4061/2011/905768.

Mason, P.W., Rieder, E., Baxt, B., 1994. RGD sequence of foot-and-mouth disease virus is essential for infecting cells via the natural receptor but can be bypassed by an antibody dependent enhancement pathway. Proc. Natl. Acad. Sci. U. S. A. 91, 1932–1936.

Pan, C.Y., Lan, X.Y., Chen, H., Yang, D.Y., Hua, L.S., Yang, X.B., Lei, C.Z., Guo1, Y.K., Zhang, B., Zhang, C.L., Kang, X.T., Wang, I.Q., 2008. A DdeI PCR–RFLP detecting a novel missense mutation of the POU1F1 gene showed no effects on growth traits in cattle. Czech J. Anim. Sci. 53, 523–527.

Robinson, T.P.P.K., Franceschini, T., Kruska, G., Chiozza, R.L., Notenbaert, F., Cecchi, A., Herrero, G., Epprecht, M., Fritz, M., You, S., Conchedda, L., See, G.L., 2011. Global Livestock Production Systems. FAO and Nairobi/ILRI, Rome, Italy/Kenya.

Sambrook, J., Russell, D.W., 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY, USA.

Singh, B., Prasad, S., Sinha, D.K., Verma, M.R., 2013. Estimation of economic losses due to foot and mouth disease in India. Indian J. Anim. Sci. 83, 964–970.

Springer, T.A., 2002. Predicted and experimental structures of integrins and ß-propellers. Curr. Opin. Struct. Biol. 12, 802–813.

Ye, S., Dhillon, S., Ke, X., Collins, A.R., Day, I.N., 2001. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res. 29 (17), E88.