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Identification of SNPs in Beta 2 Microglobulin (β2M) Gene and their Association with IgG Concentration in Neonatal Buffalo Calves

Rajni Chaudhary[1], Subodh Kumar[2]*, H.M. Yathish[3], Sivakumar A.[4], Chinmoy Mishra[5], Amit Kumar[6], Anuj Chauhan[6], B.Sivamani[7] and N.R. Sahoo[8]

[1]Division of Animal Genetics, ICAR Indian Veterinary Research Institute, Izatnagar, Bareilly - 243 122, India.
[2]Division of Animal Genetics, ICAR Indian Veterinary Research Institute, Izatnagar, Bareilly - 243122, India.
[3]Division of Animal Genetics, Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar-585401, India.
[4]Veterinary Officer, Government of Tamil Nadu, India.
[5]Division of Animal Genetics, College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture & Technology, Bhubaneswar- 751003, Orissa, India.
[6]Division of Animal Genetics, ICAR Indian Veterinary Research Institute, Izatnagar, Bareilly - 243122, Uttar Pradesh, India.
[7]Molecular Biology and Genetics, Central Institute of Brackish water Aquaculture, Chennai, India.
[8]Division of Animal Genetics, ICAR Indian Veterinary Research Institute, Izatnagar, Bareilly - 243 122, India.

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The present study was taken up to find out SNPs in Beta 2 Microglobulin (β2*M*) gene and their possible association with serum IgG concentrations of newborn buffalo calves. Forty newborn Murrah buffaloes calves were included in the present study. Polymerase chain reaction- single stranded conformational polymorphism (PCR-SSCP) technique was used to explore the polymorphism in β2*M* gene of the buffalo calves. The PCR-SSCP analysis and sequencing of different patterns obtained by silver staining of various exonic and intronic fragments revealed nine SNPs. All the identified SNPs were used to prepare the haplotypes for the calves. Fourteen different haplotypes were observed. Indirect ELISA was done to estimate IgG concentrations in serum samples of the calves. IgG levels in calf serum ranged from 0.25 to 14.88 mg/ml and mean IgG concentration in serum was estimated as 11.68 ± 0.75 mg/ml. The least square analysis of variance could not exhibit a significant effect of calf haplotype on serum IgG concentration.

Keywords: Beta 2 microglobulin, buffalo, ELISA, haplotypes, IgG, SSCP.

Calves are the future of livestock industry. Calf management plays an important role in the development of the dairy sector of the country. The success of the dairy industry depends on appropriate calf management. Calf care is not only essential for sustenance of the dairy industry but is also essential in the wake of preserving and maintaining our good quality germplasm. Important aspects in the calf rearing are health management and proper nutrition to the calves especially during the first few hours of life. These calves are born with a predetermined genetic potential, which may

* To whom all correspondence should be addressed.
Mob.: +91-9897225710;
E-mail: subkum@gmail.com

be permanently affected by management decisions implemented throughout the rearing period. It is essential that farm owners take proper care of calves so that they develop into healthy adult animals and achieve a high lifetime milk production. Proper management of young stock, particularly during the neonatal period, could markedly reduce morbidity and mortality, whereas improper management would lead to economic losses from increased cost of veterinary intervention, death losses, reduced growth and suboptimal productive and reproductive performance. Diseases of new born calf and neonatal calf mortality are the major causes of economic losses in livestock production. 25 % average early calf mortality hardly provides any chance for regular replacement of low production animals. It is roughly estimated that a calf mortality of 20 per cent can reduce net profit to 38 per cent[1]. Keeping the facts in mind like severity of pathogens on calf mortality during early period of its life and economic losses due to calf mortality on dairy industry, early immunity of neonatal calves is highly essential. The neonatal calf immunity during early period is only due to passive transfer of immunoglobulins from mother through colostrum. Health and nutrition in the neonatal calves depends on the availability of good quality colostrum in adequate quantity. The environmental as well as genetic control of passive transfer of immunity is a well established fact. Therefore, identification of these genetic causes will eventually lead a way forward to increase in neonatal calf immunity and decrease in calf morbidity and mortality. FcRns are neonatal Fc receptors which are present in neonatal calf intestine, kidneys, lungs, mammary glands and vascular endothelial cells[2]. They play an important role in absorption of IgG and are also known to prolong their half life. FcRn is a heterodimer of two polypeptides i.e., a MHC class I homolog encoded by *FCGRT* gene and beta 2 microglobulin encoded by β*2M* gene. More than 50 % cases of FPT are due to defective structure of FcRn which could be due to polymorphism in genes coding FcRn i.e., *FCGRT* and β*2M*[3]. Due to dearth of information on variants in β*2M* gene and their possible role in influencing IgG levels in buffaloes, this study was taken up to identify SNPs in β*2M* gene and their association with immunoglobulin G concentration in serum of neonatal buffaloes calves.

MATERIALS AND METHODS

Experimental animals and collection of blood

Forty new born Murrah buffalo calves, maintained at Livestock Production Management (LPM) Section of IVRI, Izatnagar, were included in the present study. Approximately, 5ml of blood was collected under sterile conditions, from the jugular vein of buffalo calves in a 15ml polypropylene centrifuge tube containing 0.5ml of 2.7% EDTA solution as anticoagulant. Additionally from new born buffalo calves, 12 to 18 hrs after the first feeding of colostrum, 3ml blood was collected in vaccutainer tubes without anticoagulant for serum isolation. These were then transported to laboratory in an icebox and serum was stored at -20^{0}C till estimation of IgG concentration.

Isolation of Genomic DNA from blood

Genomic DNA was isolated from the frozen blood samples of calves using phenol-chloroform extraction method[4] with minor modifications. The genomic DNA isolated was checked for its quality, purity and concentration by routine methods. DNA samples of good quality, purity and concentration were used for further analysis.

Amplification of different regions of β2M gene

Seven pairs of primers for amplification of both exonic and intronic regions of β*2M* gene in the DNA samples of Murrah buffaloes were designed on the basis of sequences of cattle (*Bos taurus*) in public database at NCBI (AC_000167.1) with the help of Primer3 online computer software. The primers were intended to amplify seven fragments corresponding to all the four exons (full coding regions) and three introns (partial noncoding regions) of β*2M* gene in buffalo. Sequences of primers designed for amplification of fragment are given in Table 1.

Various combinations of reaction mixture were tried to optimize the concentration of each component. The optimized concentration of the components used in the reaction mixture for amplification of fragment I through fragment VII was 2.5 µl 10 X PCR buffer (1 X), 2.5 µl dNTP mix (0.2 mM), 2.0 µl MgCl2 (1.5 mM), 0.5 µl F- primer (0.2µM), 0.5 µl R- primer (0.2µM), 0.2 µl Taq DNA polymerase (1U/25µL), 2.0 µl gDNA (80-100 ng) and 14.80 µl Nuclease free water. Samples were amplified by initial denaturation at 95°C for 3 min,

35 cycles of 95°C for 30 sec, X°C for 30 sec and 72°C for 20 sec; followed by final extension at 72°C for 10 min. The annealing temperature was 57, 59.3, 55, 55, 51, 55 and 55 °C for fragments I through VII respectively.

Identification of allelic variants

Single strand conformation polymorphism (SSCP) analysis was performed on all PCR products/DNA samples for determination of allelic variants by standard procedure with slight modifications[5], followed by sequencing. In order to optimize SSCP resolution on gel, several factors viz. amount of PCR product, denaturing solution, acrylamide concentration, percentage cross-linking, glycerol, voltage, running time, temperature etc. were tested for each fragment. Each PCR product was diluted in denaturing solution (95 % formamide, 10 mM NaOH, 0.05 % xylene cyanol and 0.05% bromophenol blue, 20 mM EDTA), denatured at 95 ^{0}C for 5 min, chilled on ice and resolved on 15 % poly- acrylamide gel. The electrophoresis was carried out in a vertical electrophoresis unit, in 1X TBE buffer. The gels were stained with silver nitrate[6] and the band patterns were scored for each amplified product. Variants detected by PCR-SSCP of seven fragments of β_2M gene corresponding to 4 exons and 3 introns were sequenced by outsourcing. Both forward and reverse sequencing were carried out for each allelic pattern which was sent as triplicate for each pattern. Resulting sequences were analysed by DNASTAR (Lasergene, USA) software. By aligning observed sequences of different patterns, the nucleotide variabilities in terms of SNPs, were identified. These SNPs were combined to form various haplotypes.

Estimation of IgG concentrations

Indirect ELISA was performed to determine the Immunoglobulin-G (IgG) concentration in serum samples of calves by kit method (Koma Biotech, Korea) with some modifications. Degree of colour development was measured as absorbance (OD) at 450nm wave length. The IgG values of known samples and their OD values were used to generate a regression equation, to predict the IgG values of unknown samples using GraphPad Prism 6 software.

Association of haplotypes with IgG concentrations

The association of β2m haplotypes with Immunoglobulin G (IgG) concentration was ascertained by PROC GLM procedure of SAS 9.3 programme. The model used was $Y_{ij} = \mu + H_i + e_{ij}$, where, Y_{ij} = j^{th} observation of IgG concentration for i^{th} haplotype; H_i= effect of i^{th} haplotype; μ = over all mean, and e_{ij} = error term attached with ij^{th} subclass.

RESULTS

SNP identification in β_2M gene of buffalo calves by SSCP analysis

Seven fragments, covering all the four coding regions as well as representative region from noncoding regions, i.e. fragment I (206 bp: full exon 1), fragment II (296 bp: partial intron 1), fragment III (287 bp: full exon 2), fragment IV (227 bp: partial intron 2 and full exon 3), fragment V (226 bp: partial intron 3), fragment VI (201 bp: initial partial exon 4) and fragment VII (405 bp: remaining partial exon 4) of β*2M* gene of calves were amplified and the amplicons were subjected to PCR-SSCP analysis to identify the allelic variants in all the calves. The fragment I (206 bp) of β_2*M* gene (encompassing partial 5' UTR region, exon1 and partial intron1) was amplified and the PCR products were subjected to SSCP analysis which revealed two alleles, A and B (Accn No. KM261591 and KM261592). The sequence analysis confirmed the presence of 16 bp 5‘ UTR (outside exon 1), 119 bp exon 1 (52 bp 5‘ UTR + 67 bp coding exon 1) and 71 bp partial intron 1. The two alleles A and B differed (C/G) at nucleotide positions 45 and lied in 5‘UTR region. This SNP viz. C45G transversion was located in untranslated region. The PCR-SSCP of fragment II covering 296 bp of intron 1 also revealed two alleles (viz. A and B, Accn No. KM261600 and KM261601). The sequence analysis confirmed 296 bp partial intron 1. The alleles A and B, differed at nucleotide positions 22, 23 and 256 in the said fragment yielding to T22A transversion, T23A transversion and G256A transition. The fragment III covering 287 bp (partial intron1, exon 2 and partial intron 2) also exhibited two alleles (*viz.* A and B, Accn no.KM261593 and KM261594). The sequence analysis confirmed the presence of partial intron 1 (10 bp), exon 2 (276 bp) and 1 bp partial intron 2. The sequence analysis revealed only one SNP at 204 nucleotide position *viz.* T204C transition which was located in exon 2. A total of 64 amino

acids were found for exon 2 of the β_2M gene in buffalo. This SNP at 204th position (T204C) did not lead to alteration of amino acid (alanine), hence, was a synonymous (silent) mutation. The fragment IV covering 227 bp (comprising of partial intron 2, exon 3 and partial intron 3) revealed monomorphic pattern indicating only one allele (A, Accn no. KM261595). Sequence analysis confirmed the presence of 146 bp intron 2 (partial), 28 bp exon 3 (14 bp coding exon 3 and 14 bp 3‘ UTR) and 53 bp partial intron 3. The fragment V (226 bp) corresponding to partial intron 3 also yielded a single allele (Accn no. KM261602). Sequence analysis confirmed the presence of 226 bp partial intron 3. The fragment VI (201 bp) corresponding to partial intron 3 and partial exon

Table 1. Primers designed for amplification of different fragments of β_2M gene in buffalo

S. No	Primer Name	Primer Sequence	Product Size
1.	Fragment 1-F	5’GAGTGCGGACTATAAAGGCGAGCG3'	206 bp
	Fragment 1-R	5’CAGGGTGCCAAGTGGGTGTGAT3'	
2.	Fragment 2-F	5’CACTGAAGCTCGGATTTCTCTT3'	296 bp
	Fragment 2-R	5’CCTACACAGGGAAAGAGCTGA3'	
3.	Fragment 3-F	5’CGCTCCTCAGGTCCTCCAAAGATT3'	287 bp
	Fragment 3-R	5’CCCCACTTAACTATCCGGGGTTGTT3'	
4.	Fragment 4-F	5’GGATCTCAGGCTGGTAGGAAAGGTC3'	227 bp
	Fragment 4-R	5’GCTGTCTGTAATGGTCCCCAGG 3'	
5.	Fragment 5-F	5’GGCTTTCCCAGCATCACTAA3'	226 bp
	Fragment 5-R	5’GGTGTCTCTGCAGAACCATGT3'	
6.	Fragment 6-F	5CCCCCAGGTTTGAAGATGCC ‘3’	201 bp
	Fragment 6-R	5’CCTCCACTGCGGCTTACTGC 3'	
7.	Fragment 7-F	5’GGTCTGTGTTGGCAGTAAGCCGC 3'	405 bp
	Fragment 7-R	5’CACTTCCCTGTTTGAACGAAGGC 3'	

Table 2. Distribution of SNPs across different regions of β_2M gene in buffalo

Fragment I (Full exon 1 and partial intron 1)	C → G, 45(5‘UTR)			
Fragment II (Partial intron 1)	T → A, 22 (intron 1)	T → A, 23 (intron 1)	G → A,256 (intron 1)	
Fragment III (Full exon 2)	T → C, 204 (exon2) GCT→GCC Ala →Ala			
Fragment IV(Partial intron 2 and full exon 3)	No SNP			
Fragment V(Partial intron 3)	No SNP			
Fragment VI(Initial partial exon 4)	No SNP			
Fragment VII (Remaining partial exon 4)	G → A, 80 (3‘UTR)	A → C, 101 (3‘UTR)	A → G, 208 (3‘UTR)	A → G, 239 (3‘UTR)

Table 3. Least square analysis of variance for effect of buffalo calf haplotype on IgG concentration in calf serum

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Haplotype	13	278.6647167	21.4357474	0.93	0.5418
Error	26	602.3501233	23.1673124		
Corrected Total	39	881.0148400			

4 also yielded one allele. Sequence analysis confirmed the presence of 7 bp intron 3 (partial), 194 bp exon 4 (partial initial). The fragment VII (405 bp) corresponding to partial exon 4 exhibited four alleles (viz. A, B, C and D; Accn no. KM261596, KM261597, KM261598 and KM261599). Sequence analysis confirmed the presence of 348 bp exon 4 (partial remaining) 57 bp 3'UTR (outside exon 4). Sequence analysis of PCR products for these four patterns also revealed four SNPs viz. G80A transition, A101C transversion and A208G transition and A239G transition. All these four SNPs were present in 3'UTR region. Sequences of all the amplified fragments conformed to the GenBank resource for β_2M gene in *Bos taurus* cattle (AC_000167.1). All these SNPs have been presented in Table 2.

Determination of haplotypes

We identified nine SNPs covering exonic and intronic regions of the β_2M gene in murrah breed of buffaloes. Before proceeding for any association study, it is imperative to make combination of these SNPs in form of haplotypes. Therefore, on the basis of SNPs, the haplotypes were prepared for all forty buffalo calves under investigation. A total of fourteen haplotypes were observed on the basis of nine SNPs viz. (C/G) (T/A)(T/A)(G/A)(T/C)(G/A)(A/C)(A/G)(A/G) were CTTGTACAA, CTTGCACAA, CTTGTGAAA, GTTGTGAAA, CTTGTGAAG, CTTGTGAGA, CTTGCGAAG CAAATACAA, GTTGTACAA, GTTGTGAGA, CAAATGAGA, CAAATGAAA, GTTGTGAAG and GAAATGAAG which were designated as H1 to H14.

Estimation of Immunoglobulin G (IgG) concentrations

Immunoglobulin G levels were estimated in serum of new born calves. The regression curve was obtained by plotting standard concentrations against absorbance at 450nm. The regression equation $y = 0.001x + 0.897$, which was able to explain 99.2 % ($R^2 = 0.992$) variation in IgG values, was used to predict the unknown IgG concentration in serum samples. IgG levels in calf serum ranged from 0.25 to 14.88 mg/ml and mean IgG concentration in serum was estimated as 11.69 ± 0.75 mg/ml.

Association of haplotypes in β_2M gene with IgG concentration

The effect of calf haplotypes was evaluated on serum IgG concentration. The least square analysis of variance revealed a non-significant effect of calf haplotype on IgG concentrations in serum (Table 3) and the least square means of IgG in various haplotypic groups were non significantly different from each other (Table 4).

Table 4. Least square means of IgG concentrations in serum with standard error (number of observations) for different haplotypes

Haplotype ID of Calves	Least square mean ± SE (n) of Serum IgG
H1	11.57 ± 1.51 (12)
H2	13.99 ± 0.00 (1)
H3	14.03 ± 0.16 (3)
H4	5.32 ± 3.90 (2)
H5	10.95 ± 1.74 (10)
H6	13.61 ± 0.02 (2)
H7	14.35 ± 0.00 (1)
H8	14.026 ± 0.37 (2)
H9	14.45 ± 0.44 (2)
H10	14.23 ± 0.00 (1)
H11	10.88 ± 0.00 (1)
H12	1.057 ± 0.00 (1)
H13	13.75 ± 0.00 (1)
H14	13.92 ± 0.00 (1)
Total	11.69 ^{NS}± 0.75 (40)

DISCUSSION

Bovine neonatal calves get their preliminary immunity through the process of passive transfer i.e. due to feeding of colostrums containing immunoglobulins (mainly IgG). Immunoglobulin concentration in milk has been found to be influenced both by genetic and environmental factors[7]. IgG can be selectively transferred from serum to milk in mammary gland and milk to blood in intestinal epithelium of neonates by the Fc receptor (FcRn) mediated mechanism[8]. The IgG's transporter receptor molecule is FcRn heterodimer of which, β_2M acts as an integral component of its cell surface expression[9-10] and in its absence, FcRn is retained in endoplasmic reticulum. Furthermore, in absence of β*2M*, IgG binding is decreased compared with that of native FcRn[11]. The β*2M*, a 6 kb gene (357 bp cds), located on 10th chromosome of bovine, is conserved across species, including mammals and

birds containing four exons and three introns. It is located outside of the MHC region. In bovines, the cds of β*2M* consist of 357 bp sequence, coding for 118 amino acid residues.

We identified nine unique SNPs located in different exonic and intronic regions of the β_2M gene in neonatal buffalo calves *(Bubalus bubalis).* Eight of these SNPs were located in non coding or intronic regions while only one was found in exon 2 of the gene though this too was a synonymous mutation. The previous study on β_2M gene in multi-breed herd of beef cattle using direct sequencing technique revealed 12 SNPs in exon 2 and 4[12]. Eleven SNPs were present in non coding region and one SNP was present in coding region of exon 2. However, this coding region's SNP was a also silent mutation and did not led to any change in amino acid. The observations on SNPs observed in murrah buffaloes in our study were in high conformity with those of above study which were observed in beef cattle. Though the SNPs reported by them were different as compared to ours. In another study, one SNP (T204C) was observed in exon 2 and flanking intron in buffalo dams[13].

It has been reported that < 10 mg/ml level indicate the failure of passive transfer (FPT) and such calves have shown greater association with morbidity and mortality as compared to those which have > 10 mg/ml of serum IgG[14-15]. According to National Dairy Heifer Evaluation Project[16], more than 40 % dairy heifer calves were found to have serum IgG < 10 mg/ml thus have a risk of FPT. In our study, only 7 out of 40 buffalo calves exhibited < 10 mg/ml thus accounted for 17.5 % of FPT cases. The greater variations observed in serum IgG in our calves are in line with other studies in which a wide range of IgG concentrations in colostrum (milking 2 to 6 hrs post calving) varying from 11 to 221 mg/ml was observed[17].

In our studies, the association between calf haplotype and serum IgG concentration could not be observed. The mean serum IgG concentration pertaining to these 14 calf haplotypes were non significantly different from each other. It may imply that either the buffalo $\hat{a}_2M$ gene may not be playing a role in determining the IgG concentrations of colostrum or serum or their might be some other important gene *viz. FCGRT* which codes for a bigger component of FcRn molecule, be playing a greater role in determining IgG concentrations of colostrum or serum. The investigation on FCGRT gene are also underway in our laboratory. Recently in another studies, three SNPs in exon 2 and flanking region and two SNPs in exon 3 and flanking introns in FCGRT gene in buffaloes were observed[18,19]. There are ample evidences that β_2M gene has been used by numerous workers as a house keeping gene (reference gene/indigenous control/ internal control) in numerous gene expression studies in a variety of tissues specially sperm[20-27] indicating that β_2M gene is stable in expression in different conditions across tissues.

On contrary, it has been reported that calves homozygous for one haplotype (out of eight observed haplotypes) of β_2M gene in multi-breed herd of beef cattle, were found to be at increased risk of failure of passive transfer[12]. The difference of our report with that of above report could be attributable to difference in species and /or the much larger sample size in beef cattle. Association between buffalo dam ß2M gene haplotypes and colostral IgG could also not be observed[28]. The lack of association in our study could also be due to the smaller sample size of buffalo calves (n=40) or it may also imply that some other non-genetic factors may be playing a greater role in determining IgG concentrations of colostrum or serum.

CONCLUSIONS

In the present investigation, we observed nine unique SNPs in neonatal buffalo calves that led to formation of 14 haplotypes. The least square analysis of variance could not exhibit a significant effect of calf haplotype on serum IgG concentration. It would also be worthwhile to study the allelic variations of the other gene *i.e., FCGRT* which codes for the heavier MHC like polypeptide of the obligate heterodimer found in the FcRn molecule with a larger sample size of buffalo calves.

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