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Research Article

GENETIC POLYMORPHISMS IN NON-CODING REGIONS OF INSULIN LIKE GROWTH FACTOR-1 GENE IN SIX INDIGENOUS DRAUGHT CATTLE OF INDIA

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Abstract: This study was undertaken to detect genetic polymorphisms in the non-coding regions of bovine insulin-like growth factor (IGF-1) gene in six indigenous draught cattle breeds *viz*., Bargur, Hallikar, Kangayam, Ongole, Pulikulum and Umblachery of Southern India. A total of 312 blood samples (52 samples from each breed) were collected and genomic DNA was isolated. Four sets of primers were designed for the amplification of the expressed regions of the IGF-1 gene along with intronic sequences on either side. The presence of eight polymorphisms was detected in the intervening sequences. The variations seen in the intronic regions were at g. 316 C>A (transversion), g. 426 G>A (transition), g. 435 G>A (transition), g. 1747 A>T (transversion), g. 1884 C>A (transversion), g. 1940 C>T (transition), g. 4707 G>A (transition) and g. 4954 C>A (transversion). Deletion of 'G' at position g. 4940 and replacement of 'C' with 'A' at position g. 56413 was found in all the six indigenous breeds. Genotyping these variations in larger number will give significant information on their role among Indian draught cattle.

Keywords: *Genetic polymorphism, Draught cattle, Insulin-like growth factor 1 gene, Non-coding region*

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Introduction

The indigenous breeds of cattle have a compact body mass, physical structure and phenotypic adaptive changes, best suited for draught animal power, needed for tropical agricultural work. At present in India around 100 million hectares of farm land are ploughed by draught animals, which form 55 percent of total cultivable area [1]. On the other hand, according to 19th Livestock Census Report (2012) [2], it becomes obvious that draught animal population in India has been steadily declining. However, critical review of literature revealed that extensive studies had been carried out on physical characteristics, work performance and biochemical parameters of working bullocks. But the work on genetic improvement of draught cattle and molecular markers related to draught power has not been attempted so far. Hence the present study was planned to characterise the bovine Insulin-like growth factor-1 (IGF-1) gene and to explore the polymorphisms of the gene involved in the main metabolic pathway related to physical performance of draught cattle. In the present research, six popular draught breeds of south India *viz*. Bargur, Hallikar, Kangayam, Ongole, Pullikulum and Umbalachery were selected. IGF-1 is considered as one of the potential candidate markers for muscle strength and muscle mass in cattle due to their role in regulation of cell proliferation and animal growth [3]. It is a polypeptide of molecular weight 7.5 kDa, built of 70 amino acids [4] and is identical in human, cattle, dogs and pigs [5]. The mature IGF-1 in cattle is expressed from a gene consisting of 4 exons (exon 1-4) and spanning more than 71 kb of genomic DNA. The gene was localized on chromosome 5 in cattle [6,7]. A recent study in human suggested that polymorphism in this gene might influence the muscle strength in response to prolonged physical exercise [8]. Thus, bovine IGF-1 gene is considered to contribute exercise tolerance in these draught breeds.

Materials and Methods

A total of 312 blood samples (52 samples from each breed) were collected from respective breeding tracts of the six breeds in sterile vacutainers, containing EDTA as an anticoagulant and stored at 4ºC till further processing. Genomic DNA was isolated using standard phenol-chloroform extraction procedure [9] with slight modifications by using DNAzol reagent for lysis and then DNA was diluted to 50 ng/µl. The purity and concentration of DNA samples were estimated by Biospectrophotometer (Eppendorf, USA). Based on the bands observed in the agarose gel and concentration determined by spectrophotometer measurement, DNA was diluted using Tris EDTA buffer in 1 in 25 or 50 or 100 dilutions to obtain the template DNA (working DNA) concentration of approximately 20 to 50 ng per µl and stored at -20ºC till further processing. Using "Primer3" online software tool (http://primer3.wi.mit.edu/), four sets of primers were designed to amplify the regions of the IGF-1 gene (1 to 262, 4735 to 4894, 56190 to 56371 and 71601 to 71821 nucleotide positions corresponding to GenBank accession No. AC_000162.1) along with introns on either sides [Table-1]. The most critical variables considered while designing the primers were primer length (18-24 bp), melting temperature (55ºC to 80ºC), specificity, complementary primer sequence, GC content (40 percent to 60 percent) and 3'-end sequences. PCR was performed by following the protocol given in [Table-2]. The PCR amplicons were analysed on a 2% agarose gel and bands were documented. The bands developed were observed in a GelDoc (Bio-Rad,USA) system. The amplicons were sequenced in both forward and reverse directions at M/s. Ocimum Biosolutions, Hyderabad.

Table-3 Genetic polymorphisms found in intronic regions of IGF-1 gene between *Bos taurus* and *Bos indicus* cattle

Locus (position in bp)	*Bos taurus* cattle	*Bos indicus* cattle					
		Bargur	Hallikar	Kangayam	Ongole	Pulikulum	Umblachery
Parts of intron 2							
316	C	AC	AC	AC	AC	AC	AC
426	G	AG	AG	AG	AG	AG	AG
435	G	AG	AG	AG	AG	AG	AG
1747	A	AT	AT	AT	AT	AT	AT
1884	C	AC	AC	AC	AC	AC	AC
1940	C	CT	CT	CT	CT	CT	CT
4707	G	AG	-	AG	AG	AG	AG
Parts of intron 3							
4940	G	Deletion of G in all breeds					
4954	C	AC	-	AG	AG	AG	AG
Parts of intron 4							
56413	C	Replaced by A in all breeds					

(Dash indicates no polymorphism detected)

Table-4 Genotype frequency of variations found in intronic regions of IGF-1 gene in different breeds of *Bos indicus* cattle

Locus (Position in bp)	Genotype	Cattle breeds and genotype frequency					
		Bargur	Hallikar	Kangayam	Ongole	Pulikulum	Umblachery
Parts of intron 2							
316	CA	0.20	0.00	0.50	0.16	0.50	0.00
	CC	0.80	1.00	0.50	0.83	0.50	1.00
426	GA	0.00	0.00	0.50	0.00	0.50	0.40
	GG	1.00	1.00	0.50	1.00	0.50	0.60
435	GA	0.00	0.00	0.50	0.16	0.50	0.60
	GG	1.00	1.00	0.50	0.83	0.50	0.40
1747	AT	0.00	0.00	0.40	0.00	0.40	0.00
	AA	1.00	1.00	0.60	1.00	0.60	1.00
1884	CA	0.50	0.00	0.60	0.00	0.60	0.00
	CC	0.50	1.00	0.40	1.00	0.40	1.00
1940	TC	0.00	0.00	0.60	0.00	0.60	0.00
	TT	1.00	1.00	0.40	1.00	0.40	1.00
4707	GA	0.37	-	0.00	0.00	0.50	0.14
	GG	0.62	-	1.00	1.00	0.50	0.85
Parts of intron 3							
4954	CA	0.37	-	0.00	0.00	0.50	0.14
	CC	0.62	-	1.00	1.00	0.50	0.85

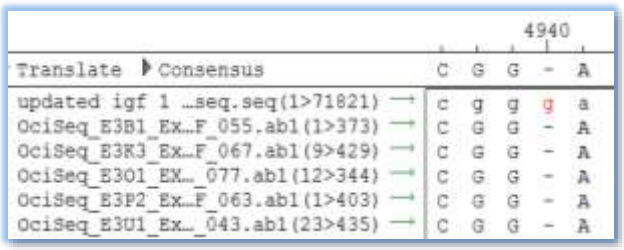
(Dash indicates no polymorphism detected)

Table-5 Allele frequency of variations found in intronic regions of IGF-1 gene between *Bos taurus* and *Bos indicus* cattle

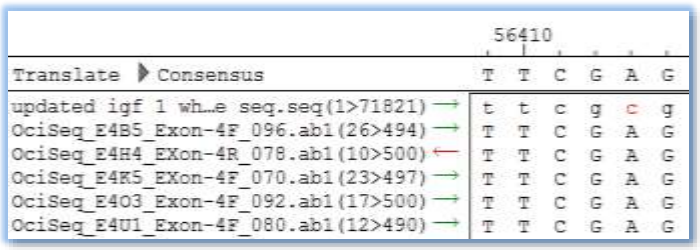
Locus (position in bp)	Allele	Cattle breeds with allele frequency					
		Bargur	Hallikar	Kangayam	Ongole	Pulikulum	Umblachery
Parts of intron 2							
316	A	0.10	0.00	0.25	0.09	0.25	0.00
	C	0.90	1.00	0.75	0.91	0.75	1.00
426	A	0.00	0.00	0.25	0.00	0.25	0.20
	G	1.00	1.00	0.75	1.00	0.75	0.80
435	A	0.00	0.00	0.25	0.09	0.25	0.30
	G	1.00	1.00	0.75	0.91	0.75	0.70
1747	A	1.00	1.00	0.80	1.00	0.80	1.00
	T	0.00	0.00	0.20	0.00	0.20	0.00
1884	A	0.25	0.00	0.30	0.00	0.30	0.00
	C	0.75	1.00	0.70	1.00	0.70	1.00
1940	C	1.00	1.00	0.30	1.00	0.30	1.00
	T	0.00	0.00	0.70	0.00	0.70	0.00
4707	A	0.20	-	0.00	0.00	0.25	0.08
	G	0.80	-	1.00	1.00	0.75	0.92
Parts of intron 3							
4954	A	0.20	-	0.00	0.00	0.25	0.08
	C	0.80	-	1.00	1.00	0.75	0.92

(Dash indicates no polymorphism detected)

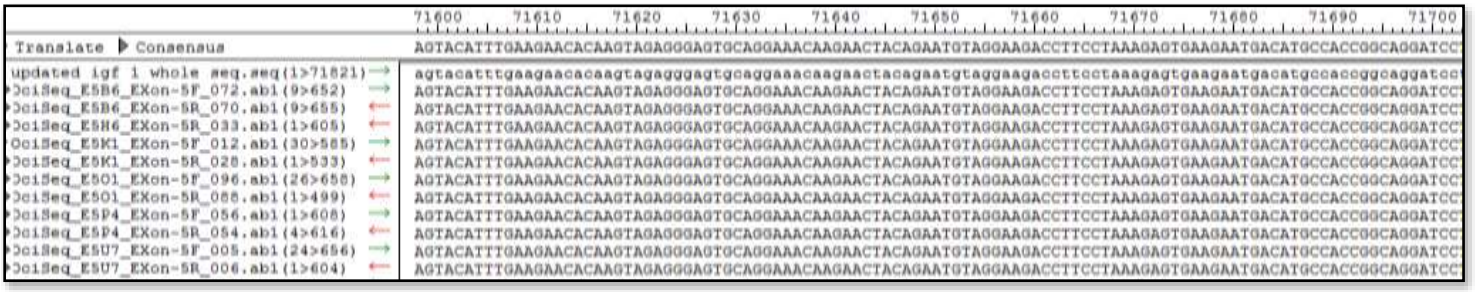
The instrument used for sequencing was ABI 3730XL DNA analyser (Applied Biosystems, USA). The variations in sequences among the six cattle breeds and individual animals within a breed were determined using DNA Lasergene Version 2.1 software. The *.ab1 files obtained were fed to "Seqman module" of Lasergene software for multiple sequence analysis. The *Bos taurus* sequence was considered as the reference sequence and was aligned with the query sequences of *Bos indicus*. This software created the consensus sequence and highlighted the polymorphisms, which were verified by base calling using chromatogram. The variation position was noted down from the reference sequence and was marked as a polymorphism.



Deletion of 'G'at position 4940 *in Bos indicus* cattle (part of intron 3)



Replacement of C from A at position 56413 in *Bos indicus* cattle (part of intron 4)



No polymorphism between *Bos taurus* and *Bos indicus* cattle extending after exon 4

Fig-1 Major differences and consensus in the sequences between *Bos taurus* and *Bos indicus* cattle

Table-1 Primer sequences designed for amplifying IGF-1 gene

Region	Primer Sequence (5'-3'end)		Annealing Temperature (ºC)
1	Forward	ttt gcc aga aga ggg aga ga	62.0
	Reverse	caa gcc ctg aag aag tgg ag	
2	Forward	tag cat gat gcc aag acc tg	53.8
	Reverse	gct cgc att aag gtg agg aa	
3	Forward	gaa aaa cct ggg agg gtc a	59.9
	Reverse	cct ctc agg gga gaa tgg a	
4	Forward	cca tgc cat caa ggg aaa	52.4
	Reverse	caa gcc tgc tga atg aat g	

Table-2 PCR protocol for IGF-1 gene amplification

Step	Process	Temperature	Duration
1	Initial denaturation	95ºC	5 min
2	Denaturation	95ºC	45 sec
3	Annealing : Exon 1	62.2ºC	1 min 30 sec
	Exon 2	53.8ºC	40 sec
	Exon 3	59.9ºC	1 min 30 sec
	Exon 4	52.4ºC	45 sec
4	Extension : Exon 1	72ºC	1 min 15sec
	Exon 2	72ºC	40 sec
	Exon 3	72ºC	1 min
	Exon 4	72ºC	1 min 15 sec
5	Back to steps 2 to 4	35 cycles	
6	Final extension	72ºC	10 min
7	Hold	4ºC	Until the samples are removed

Results and Discussion

It is well established that gene transcription is extensively and co-ordinately regulated. Although introns were known to carry regulatory sequences, they may not have a direct involvement in the regulation of transcription of highly expressed genes; however, systematic differences in motif distribution suggested that introns play a key role in the rate of their transcription [10]. Considering this piece of information, five intronic regions of IGF-1 gene were amplified while generating the exonic amplicons of various sizes. Altogether, four exons of IGF-1 gene were amplified generating amplicons of sizes 607, 454, 518 and 671 bp covering introns on both the sides as the actual sizes of exons were 262, 160, 182 and 221 bp only (NCBI; GenBank ID 281237). Nucleotide sequence of 89 bp was amplified upstream of exon 1 which formed the part of intron 1 or promoter region. However, no difference in the sequences between *Bos taurus* and *Bos indicus* cattle was observed. In intron 2, three parts were amplified with the fragment sizes of 256 bp (*i.e.*, immediately after exon 1), 180 bp (preceding exon 2) and 410 bp (between exons 1 and 2). The intervening sequences of intron 2 exhibited highest polymorphism, both between *Bos taurus* and *Bos indicus* and among various breeds of south Indian cattle. The variations seen were g. 316 C>A (transversion), g. 426 G>A (transition), g. 435 G>A (transition), g. 1747 A>T (transversion), g. 1884 C>A (transversion) and g. 1940 C>T (transition). The difference in parts of intron found between *Bos taurus* and *Bos indicus* cattle; and among indigenous breeds of cattle are expressed in [Table-3]. The varying genotype and allele frequencies are given in [Table-4] and [Table-5]. Hallikar was the only cattle breed which did not show any polymorphism in any of the positions. Even though, the second part of intron 2 was considered as an expressed region of partial cds [11], the sequence of this region obtained from *Bos indicus* cattle aligned with the intronic position in the updated IGF-1 sequence of *Bos taurus* cattle. Amplification of parts of intron 3 yielded the size of products as 122 bp (exon 2+) and 172 bp (preceding exon 3). Three different variations were found in the first product at positions g. 4707 G>A (transition), g. 4954 C>A (transversion) and deletion of G at position g. 4940 in zebu cattle. This kind of polymorphism was observed in Bargur, Ongole, Pulikulum and Umblachery breeds. But in Kangayam, except deletion of 'G' at position g. 4940, no other polymorphism was detected. The delG at position g. 4940 and replacement of 'C' with 'A' at position g. 56413 found in all indigenous breeds suggest that these variations would have resulted since the divergence of both *Bos taurus* and *Bos indicus* cattle at the time of evolution. Two different PCR products were obtained *viz.* 230 bp and 228 bp fragments, aligned between positions g. 56371 and g. 56601 (immediately after exon 3; exon 3+); and between g. 71473 and g. 71601 nucleotides in the whole gene (preceding exon 4). The only variation found was replacement of 'C' by 'A' in all breeds of *Bos indicus* cattle at position g. 56413. A region of 243 bp was amplified extending after exon 4 (exon 4+) displaying no polymorphisms. This region exhibited greatest homology between sequences of *Bos taurus* and *Bos indicus* cattle. A recent study was conducted to study the polymorphisms in the coding sequences of the gene and overall six SNPs in four exons of IGF-1 gene were found to be the characteristics of *Bos indicus* cattle [12].

Conclusion

Overall eight polymorphisms in the intervening sequences of IGF-1 gene were found to be characteristics of *Bos indicus* cattle. The deletion of 'G' at position g. 4940 and replacement of 'C' with 'A' at position g. 56413 found in all the six breeds of zebu cattle suggest that these variations had resulted due to divergence of both *Bos indicus* and *Bos taurus* cattle. A candidate gene approach would pave the way for selecting animals with better draught ability, *i.e.*, selection based on molecular tool. Further, it will also help in implementing rational decisions for conservation and improvement of our treasured animal genetic resources.

Application of research: This study is first of its kind in India to characterise the IGF-1 gene in *Bos indicus* cattle and to explore the polymorphisms of IGF-1 gene involved in the main metabolic pathway related to physical performance of draught cattle. A candidate gene approach would pave the way for selecting animals with better draught quality, *i.e*., selection based on molecular tool. Further, it will also help in implementing rational decisions for conservation and improvement of our treasured indigenous genetic resources.

Research category: Animal Genetics

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