

## cDNA CLONING AND SEQUENCING OF INSULIN-LIKE GROWTH FACTOR 1 IN IRANIAN CATTLE

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### Summary

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The aim of this study was to clone and sequence the cattle IGF-1 gene in *E. coli*, done for the first time in Iran. The cDNA that encodes cattle IGF-1 was isolated from total mRNA of the bovine liver and amplified by PCR using IGF-1 specific PCR primers. Sequencing of DNA is the process of determining arrangements of nucleotides on a fragment of DNA. Cycle sequencing is a procedure used to increase the DNA sequencing sensitivity process and allow the use of very small quantity of DNA starting material. Recombinant DNA technology had revolutionised genetic and biotechnology science in recent years and helped us to find and determine some specific and useful genes. We cut DNA at specific location and inserted this specific gene into a plasmid that will re-introduce it into a bacterial cell. When the bacteria genomes multiply, the plasmids multiply as well, creating many copies of the specific gene. Since bacteria multiply very quickly, large numbers of our specific gene can be produced in the laboratory for further analysis and application.

**Key words:** cDNA cloning, IGF-1, Iranian cattle

### INTRODUCTION

Enlargement in animals is controlled by GH that acts on bones and muscles growth and is mediated by insulin-like growth factor 1 (IGF-1). The *IGF-1* and growth hormone (*GH*) genes are candidates for growth in cattle. These effects result from direct action of IGF-1-mediated action motivating cell proliferation and metabolic processes related to protein deposition and GH on the cellular proliferation and nutrients (Pereira *et al.*, 2005; Georgiev, 2008). In addition, IGF-1 plays an important role in cattle follicular growth, embryo viability and oocyte efficiency (Velazquez *et al.*, 2009). Associations between the stage of follicle development and follicular fluid concentration of total

IGF-1 have been observed in many species, including sheep and cattle (Khairy *et al.*, 2007). Candidate genes determine biological functions related to the development or physiology of a significant trait. Such genes can encode ideal proteins or parts of a regulatory or biochemical pathway affecting the expression of the trait (Pereira *et al.*, 2005).

Nowadays economic traits (genetic variation) in cattle have been used by breeders to choose the ideal animal in a breed. Crosses between breeds allow the formation of new breeds, selected for best traits including adaptation to the environment (Pereira *et al.*, 2005). Specifically, IGF-1 is expressed in infantile life and is

entirely synthesised in the liver controlled by growth hormone. It appears that the IGF may have both endocrine and paracrine performance of intercellular connection in controlling the growth of many tissues *in vivo*. The liver is the major endocrine source of IGFs (Zahran & Aboul-Soud, 2007; Georgiev, 2010). The *IGF-1* gene regulates growth, differentiation and the maintenance of differentiated function in several tissues and particularly cell types of mammals via binding to a group of particular membrane-associated glycoprotein receptors (Fatima *et al.*, 2009). IGF-1 orders the growth of many kinds of mammalian cells. Evidence indicate that IGFs are involved in numerous biological processes, like development, metabolism and growth and muscle fibre diameter (Zahran & Aboul-Soud, 2007; Lei Yao *et al.*, 2009). IGF-1 is a mediator of many biological effects; for example, it increases the absorption of glucose, stimulates myogenesis, inhibits apoptosis, participates in the activation of cell cycle genes, increases the synthesis of lipids, stimulates the production of progesterone in granular cells, and intervenes in the synthesis of DNA, protein, RNA, and in cell proliferation (Reyna *et al.*, 2010). IGF-1 can act through endocrine, paracrine, and autocrine mechanisms. The *IGF-1* gene in humans consists of six exons (Duncan, 2011). In humans, goats, chickens, pigs, and rats the nucleotide sequence of IGF-1 is about 70–90 kb. Exon number differs between species (Reyna *et al.*, 2010). A single gene locus for *IGF-1* has been mapped to the long arm of human chromosome 12 and bovine chromosome 5 (Zahran & Aboul-Soud, 2007). Bovine IGF-1 is a 70-amino acid, single chain basic polypeptide, with molecular mass of 7,649 Da. The amino acid sequence is 96% conserved and few polymorphisms

are expressed. The existence of a microsatellite at the promoter region of this gene in humans, cattle and horses allows studying genetic variations related to this locus (Pereira *et al.*, 2005; Zahran & Aboul-Soud, 2007; Zych *et al.*, 2007). The bovine cDNA is 93% identical to the human sequence (Zahran & Aboul-Soud, 2007).

The transgenic technologies have brought a new aspect into *in vivo* observation of gene function. It is now possible to insert new functional genes, modify and inactivate the existing ones in living organisms. The present animal experiments with gene transfer will undoubtedly also help to future gene therapy of human diseases (Khairy *et al.*, 2007). Cloning genes is now a technically uncomplicated process. Cloning uses recombinant DNA techniques. This technology allows scientists to find individual genes, cut them out, and insert them into the genome of another organism (Doosti *et al.*, 2011). DNA sequencing is the process of determining the order of nucleotides (A, T, C, and G) along a DNA strand. The sequence of the whole DNA chain can be determined by this method (Sanger *et al.*, 1977). When chain completion sequencing is performed manually, each of four tubes contains a dissimilar type of terminator base for labelling the DNA fragments as they are made. All of the four reactions are electrophoresed in a distinctive lane of a gel, and a X-ray film is used to detect the fragments (Sanger *et al.*, 1977).

The purpose of the present study was to identify and isolate Iranian native cattle *IGF-1* gene. In addition we have cloned and sequenced IGF-1 to compare it to the results reported from other parts of the world (Khairy *et al.*, 2007; Zahran & Aboul-Soud, 2007).

## MATERIALS AND METHODS

Fresh bovine liver was obtained from Iranian native cattle carcasses in Chaharmahal Va Bakhtiari province, southwest Iran. The liver stored at  $-80^{\circ}\text{C}$  until used. Total RNA was isolated from bovine liver tissue using a Qiagen RNA extraction kit (Qiagen, Ltd., Crawley, UK). RNA was reverse transcribed to cDNA with a first strand cDNA synthesis kit (Fermentas, Germany) according to the manufacturer's protocols. Bovine IGF-1 cDNA was amplified using the specific primers for the coding region of bovine IGF-1 cDNA and primers were designed according to the published sequence for insulin like growth factor cDNA of the cattle (accession number: NM\_001077828.1). Primer sequences were the following: the forward primer was BIGF-F: 5'- ATAGGATCC ACCAATTATTTAAGTGCTGCT-3' and the reverse primer was BIGF-R: 5'- ACTAAGCTTTGTCATTCTTCACTCT TTAGG-3'. Restriction endonuclease sites of *Bam*HI and *Hind*III (underlined nucleotides in the above sequences) were integrated into the 5' end of primers BIGF-F and BIGF-R, respectively. In order to amplify bovine insulin like growth factor cDNA, PCR was performed in a 25  $\mu\text{l}$  total volume containing 2.5 microliter of  $10\times$  PCR buffer, 1  $\mu\text{M}$  of each primer (BIGF-F and BIGF-R), 2 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  dNTP, 1  $\mu\text{g}$  of template cDNA, 1 unit of *pfu* DNA polymerase (Roche Applied Science). The 30-cycle amplification was performed in a thermal cycler system (Palm Cycler Gradient, Australia) with the following programme:  $94^{\circ}\text{C}$  for 60 s,  $60^{\circ}\text{C}$  for 60 s,  $72^{\circ}\text{C}$  for 60 s. A final 7 min extension was performed at  $72^{\circ}\text{C}$ .

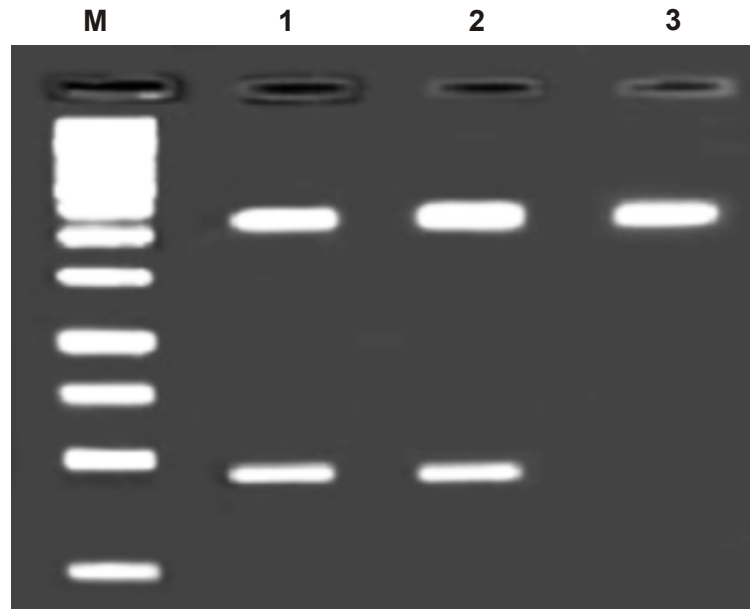
Agarose gel electrophoresis was subsequently performed by using 1% agarose gel in  $1\times$  TBE buffer to verify and visualise the product size, which was stained by

ethidium bromide on UV transilluminator. The agarose gel slice containing the relevant bovine IGF-1 cDNA fragment was excised and purified by gel extraction kit (Bioneer, Korea) according to the manufacturer's recommendation. Cloning and preparation of bovine TOPO-IGF-1 cDNA was cloned using T/A cloning technique. T/A cloning is one of the most popular methods of cloning the amplified PCR product using *pfu* and other polymerases. Bovine IGF-1 cDNA fragment ligated to the T-Vector using TOPO T/A cloning kit (pCR8/GW/TOPO, Invitrogen) according to the manufacturer's instructions to obtain TOPO-IGF-1. *Bam*HI/*Hind*III restriction analysis and PCR technique were used to confirm gene cloning. Finally, the recombinant plasmid (TOPO-IGF-1) was sequenced by specific primers and the Sanger sequencing method (Macrogen, Korea) (Sanger *et al.*, 1977).

## RESULTS

The cDNA was successfully prepared from total RNA obtained from bovine liver tissue. Polymerase chain reaction amplification with bovine IGF specific primers generated a 489 bp fragment for identification of IGF-1, which was cloned through the T/A cloning technique in a T/A vector (pCR 8/GW/ TOPO Vector).

Fig. 1 shows recombinant plasmids after digestion. A 2,817 bp large fragment is related to the TOPO vector and the 489 bp fragment is the cDNA band. Chemical competent cells of *E. coli* were transformed with TOPO-IGF recombinant plasmid. Plasmid purification and *Bam*HI/*Hind*III restriction endonuclease digestion of TOPO-IGF recombinant plasmid, shown to be true the correction of IGF-1 cDNA cloning. Recombinant plasmids after digestion showing the 489 bp



**Fig. 1.** Analysis of TOPO-GH recombinant vector using restriction endonuclease enzyme. Line M – 1 kb DNA ladder (Fermentas, Germany), lines 1 and 2 – TOPO-IGF-1, line 3 – TOPO vector without IGF-1.

fragments is the IGF-1 cDNA band and the 2,817 bp large fragment is related to the TOPO vector. The native Iranian cattle's cDNA was successfully sequenced and compared with IGF-1 nucleotide sequences of other records in the GenBank using basic local alignment search tool (BLAST) software; the pointing out of similarities and differences showed 100% homology with the published IGF sequences of Iranian native bovines (Accession number: NM\_001077828.1). The sequenced IGF-1 cDNA was compared with IGF-1 nucleotide sequences of other records and both of them were the same. The result of this study indicates that IGF-1 gene is one of conserved gene in the bovine populations around the world.

## DISCUSSION

The most common belief in animal breeding is that variation in quantitative features such as lactation or growth is restricted by many genes. Each of these genes has a small effect. However, few genes may be responsible for a relatively large ratio of genetic variation. Genes entangled with the biology of a trait of interest are candidates for studies, and can be taken into account as "candidate genes". It was suggested that genes coding for hormones and factors including GH, its receptor GHR, transcription factor STAT5, and insulin-like growth factors 1 and 2 (Siadkowska *et al.*, 2006). Recombinant DNA technology is a new method that has acquired popular attention in the preceding decade. This method permits us

to find particular genes, and put them into the genome of another organism. Recombinant DNA technology has implementations in nutrition and health. For example, it is used to create pharmaceutical products like human insulin or is used to instruct positive characteristics to plants to increase nutritional content yield and improve their nutritional content (Doosti *et al.*, 2012). Recombinant DNA technology works with restriction enzymes, which cut DNA at specific location. We inserted the cut-out gene into a plasmid which was then re-introduced into a bacterial cell. When the bacterial genomes multiply, the plasmids multiply as well, creating many copies of the specific gene. Since bacteria multiply very quickly, large numbers of the gene can be produced in the laboratory for further analysis and application (Doosti *et al.*, 2012). IGF1 is located on chromosome 5 of bovine, which contain at least 73 quantitative trait loci (QTL). Due to the high QTL number nearby IGF1, this chromosomal region has been evaluated not only in different species of domestic animals but also between diverse breeds for example in cattle (Reyna *et al.*, 2010). Associations were investigated among a single nucleotide polymorphism (SNP), the C/T transition at position -472 (RFLP-*SnaBI*) in the 5'-noncoding region of the *IGF-1* and milk and meat production characters in cattle (Siadkowska *et al.*, 2006).

In this work, we put to use the power of PCR technique in order to produce an Iranian cattle *IGF-1* cDNA (Khairy *et al.*, 2007). The 4.2 kb sequence product was determined and had an open reading frame encoding 1367 amino acids with 95.2 and 98.1% sequence similarity to the rat and human *IGF-1*, respectively (Khairy *et al.*, 2007).

Standard PCR was also done to eliminate false positives due to DNA contaminants. We particularly used *Pfu* DNA polymerase as it has a natural 3'→5' exonuclease activity with a proofreading function. It is a concerted enzyme in applications like cloning and protein expression requiring high conformity PCR, because it demonstrates the lowest error rate of any commercially available thermostable DNA polymerase (Khairy *et al.*, 2007). A 489 bp segment of *IGF-1* gene was amplified. IGF sequencing was performed to detect any mutation that might be present. The analysis of sequence of the *IGF-1* region in the cattle revealed no sequence variation. It indicated that polymorphism revealed by SSCP could be independent of sequence variation (Fatima *et al.*, 2009). Candidate genes play a known biological role related to the physiology or development of an essential trait. Similar genes can encode structural proteins or a member in a biochemical or regulatory pathway affecting the expression of the characteristic. If a particular haplotype can be defined at this candidate gene that can be related to milk production, protein and fat content, it would be rendered available as a valuable genetic resource for improvement of these cattle (Fatima *et al.*, 2009). Making many therapeutic proteins is made possible by molecular technologies used for the production of necessary proteins, including antibodies, blood products, cytokines, growth factors, hormones, recombinant enzymes and human and veterinary vaccines. The TOPO-IGF plasmid that was generated in this study is used for sub-cloning and production of recombinant growth hormone protein. This way, it can regulate differentiation including the maintenance of differentiated function in numerous tissues and in specific cell types and also, stimulate the

anabolic and mitogenic activity of growth hormone in various tissues.

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