# PCR AMPLIFICATION AND ANALYSIS OF VARIATION IN GROWTH HORMONE GENE OF CETARTIODACTYLA

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**Abstract**

**Background:** The sequence of growth hormones (GHs) is generally conserved in mammals but short bursts of rapid changes occur during evolution of primates and artiodactyla. This has led to marked differences in primary structure and biological specificity in cetartiodactyla. Present study was done to amplify and further explore GH evolution in cetartiodactyla.

**Methods**: Buffalo placental tissue was collected from Lahore, Pakistan and stored at -20°C. Placenta was homogenized and RNA was extracted and analyzed on horizontal agarose gel electrophoresis. RT-PCR (reverse transcription polymerase chain reaction) was performed by using oligo(dT) primers. GH gene was amplified by PCR and analyzed on agarose gel electrophoresis. PCR product was purified and sent for sequencing. Sequence alignment was done using bioinformatics tools.

**Results**: GH gene was successfully amplified. Silent mutations in the sequence of *Bubalus bubalis* GH were compared with the reported sequence of bovine GH and variations were found from reference sequence.

**Conclusion**: In conclusion, GH gene has variation in sequence during evolution due to different factors. A total of 10 variations were observed in the forms of transitions, transversions and deletion as compared to reference sequence.

## Introduction

Growth hormone (GH) is an anabolic hormone that is synthesized and secreted by the somatotropin cells of the anterior lobe of the pituitary gland. It is a member of the GH gene family, which includes prolactin and the placental lactogens (Ayuk *et al*, 2006). GH is a protein hormone of about 190 amino acids. It is a major participant which controls several complex physiologic processes, including growth and metabolism (Siddle *et al*, 1990)**.** GH is also of considerable interest as a drug used in both humans and animals. GH serves many other metabolic functions, in addition it has proven to be of great importance in farm animals. Exogenous supply of GH has a positive impact on the production of milk and meat with an increase in growth rate (Bauman *et al*, 1992).

The major isoform of the human growth hormone is composed of 191 amino acids with 2 disulphide bridges and 4 alpha helices arranged in anti parallel distinctive manner (Sami, 2007). Despite marked structural similarities between GHs from different species, only human and primate GHs have significant effects in humans. In mammals the structure of pituitary GH is generally strongly conserved, which indicated a slow basal rate of molecular evolution. However, on two occasions, during the evolution of primate and of artiodactyls, the rate of evolution has increased dramatically (25-50 fold) so that the sequences of human and ruminant GHs differ markedly from those of other mammalian GHs (Lioupis *et al*, 1997). The term Cetartiodactyla, coined by merging the name for the two orders, Cetacea and Artiodactyla, into a single word, reflects the idea that whales evolved from within the artiodactyls.

It has been observed that the bovine GH gene is 1.8 kb long and consist of five exons interrupted by four introns (Woychik *et al*, 1982). It has been described that presence of an alternatively processed species of bovine GH mRNA from which the last intron (intron D) has not been removed by splicing. The variation exists due to the miss-splicing of the $4^{th}$ intron. It was investigated that $4^{th}$ intron containing bovine GH mRNA is found, which represents that this mRNA species is translated into a polypeptide. This may be due to that the splice donor and acceptor site of $4^{th}$ intron is not behaving and because of the absence of the appropriate sequence in exon (mutation of 13-15 amino acid), splicing event does not occur (Sun *et al*, 1993).

Present study was done to find out the variations in the sequence of GH gene in the local species of *Bubalus bubalis*. This report explains the RNA isolation, RT-PCR amplification of specific genes, purification of PCR product, sequencing and analysis of GH gene.

## Materials and Methods

**RNA Extraction:** Total RNA was isolated from the placental tissue, collected from a freshly slaughtered buffalo. The sample was collected from a local (Lahore) slaughter house. RNA extraction from placental tissue was done using Tri-Reagent according to the manufacturer's instructions. Briefly, 100mg tissue was ground in 1ml of Tri Reagent and homogenized. The sample was transferred into 1.5ml tube and incubated at room temperature for 15 min. 200µl of chloroform was added and then shaken vigorously. The mixture was incubated for 15 min at room temperature and centrifuged at 12,000 g for 15 min at 4$^{o}$C. Aqueous phase was taken and RNA was precipitated by adding 500µl of isopropanol and incubated for 10 min followed by centrifugation at 12,000g for 8 min at 4$^{o}$C. The supernatant was removed and washed the RNA pellet with 75% ethanol and then centrifuged at 7,500g for 5 min at 4$^{o}$C. The ethanol was removed and briefly air-dried the pellet for 3 min and then dissolved the pellet in 50µl nuclease free water. Aliquots of 10µl RNA were prepared and stored at -80$^{o}$C and analyzed on 1.2% agarose gel electrophoresis.

**Primer designing and reverse transcription polymerase chain reaction (RT-PCR):** The nucleotide sequence of bovine GH is available at NCBI with accession number M57764 M28453 (http://www.ncbi.nlm.nih.gov/). The sequence of bovine GH encoding mature peptide is 2856bp in length. Restriction map of the gene was checked by using NEB cutter. Properties of primers (Tm, GC content, Length, Molecular Weight etc.) were checked by "Oligonucleotide properties Calculator" (http://www.basic.northwestern.edu/biotools/OligoCalc.html).
The set of primers used for the amplification of *B.bubalis* DNA is as follows:

**GH-F: Forward: 5'-** TCTCAAGCTGAGACCCTGTGT**-3'**
**GH-R: Reverse: 5'-**GGCCAAATGTCTGGGTGTAGA**-3'**

To prepare cDNA from mRNA, RT-PCR was performed by using the enzyme reverse transcriptase and oligo-dT primers. For single-stranded cDNA synthesis, 3 µl Template buffalo mRNA, 1 µl of oligo dT primer and 7 µl of nuclease free water were added together and incubated for 5 min at 70 °C and then rapidly chilled on ice followed by addition of 4 µl of 5X reaction buffer and 2 µl of dNTPs and incubated for 5 min at 37 °C, after this 1 µl (200 units) reverse transcriptase enzyme was added and incubated at 42°C for 60 min and then at 72°C for 10 min.

**PCR amplification, purification, sequencing and alignment:** PCR reaction mixture (2µl cDNA, 5 µl PCR buffer, 4 µl $MgCl_2$, 2 µl forward and reverse primers, 2.5 µl dNTPs mixture, 1 µl Taq DNA polymerase and nuclease free water was added to make total mixture up to 50 µl) was prepared and PCR was subsequently carried out according to these conditions: initial denaturation (94°C for 1min), denaturation (94°C for 30 sec), annealing (60°C for 30 sec), extension (72°C for 30 sec), for 35 cycles, final extension (72°C for 2 min), cooled and hold (4°C). PCR product was size-fractioned on agarose gel.

The amplified products were purified using rapid PCR purification kit (Marligen, USA). 200 µL of binding solution (H1) (concentrated guanidine HCl, EDTA, Tris-HCl and Isopropanol) was added to the amplification reaction and mixture was applied to a spin cartridge containing silica-based membranes where the double stranded DNA was selectively adsorbed. DNA polymerase, buffer, unreacted primers and dNTPs were removed with 500 µL of alcohol-containing wash buffer (H2) (NaCl, EDTA, Tris-HCl). DNA was eluted in 25 µL Tris-EDTA buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA) at 65°C.

The purified PCR products were sequenced on CEQ8800 DNA sequencer (Beckman Coulter, USA). Alignment was done using bioinformatics software clustal W, version 1.83 multiple sequence alignment computer program (http://www.ebi.ac.uk/Tools/msa/tcoffee/). Bovine GH Sequence with accession number of M57764 M28453 was used as reference sequence.

## Results

**RNA Extraction:** Total RNA was extracted from placental tissue and run on the gel. The RNA was successfully extracted and the band of 28S and 18S RNA can be clearly visualized on agarose gel (Fig. 1)

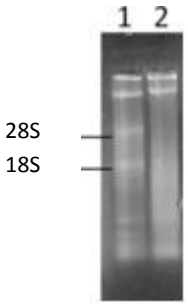


**Fig. 1. 1.2% agarose gel of total RNA isolated from placental tissue of *Bubalus bubalis***

**RT-PCR and PCR amplification of GH gene:** RT-PCR was performed using oligo-dT primer to prepare cDNA which was amplified by PCR and the product of 450bp was obtained which was analyzed on agarose gel electrophoresis (Fig. 2). The PCR product of GH gene was further confirmed by running native-PAGE and band of desired size is clearly shown (Fig. 3)

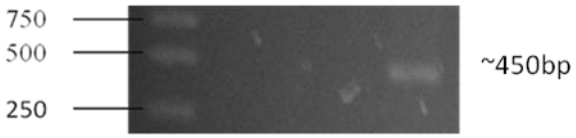


**Fig. 2. Agarose gel showing ladder of Fermantas (1Kb) and growth hormone gene PCR product of~ 450bp.**

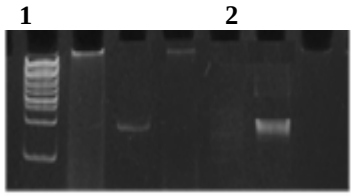


**Fig. 3. Native-PAGE of PCR product of growth hormone gene. 1 showing 1 Kb ladder, 2 showing PCR products of GH gene.**

**Sequencing and alignment:** DNA sequencing results were obtained and on analysis by alignment software variations in sequence of GH were observed at many different points. A total of 6 transitions, 3 transversions and 1 deletion were observed when we compared the sequencing results with reference sequence (Fig. 4, Table. 1). All the observed mutations were silent mutations that do not change the amino acid sequence of protein.

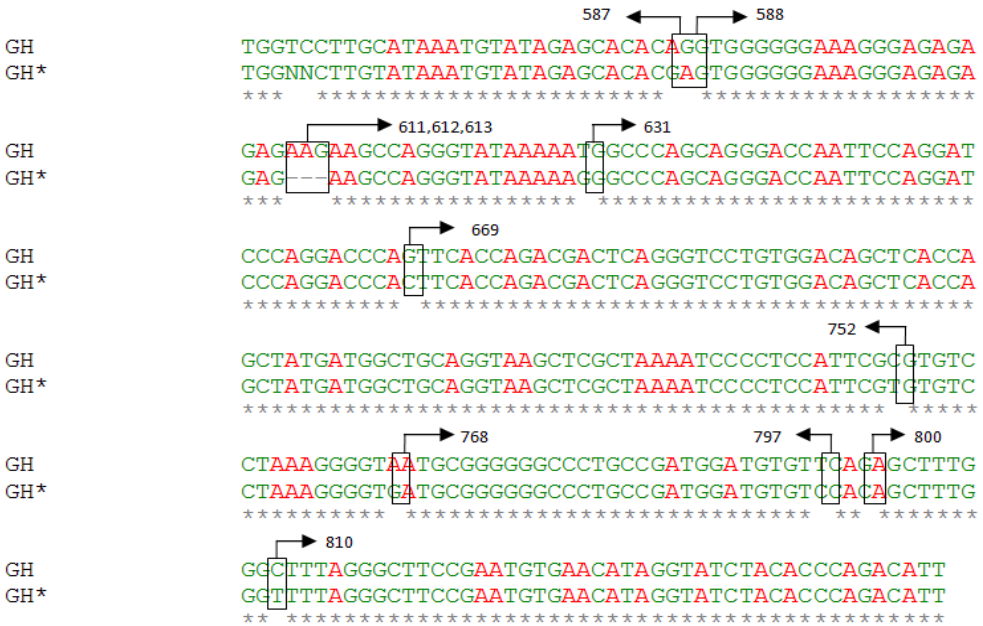


**Fig. 4. Clustal W (1.83) multiple sequence alignment of GH gene**

GH: Original Reference sequence (Bovine GH)
GH*: Data after Sequence (*B.Bubalis* GH)

**Table 1. The alignment results showed the replacement (Transition/Transversion) and deletions in GH gene of *Bubalus bubalis*.**

| S.No. | Accession No. | Position | Replacement | Tansition/Transversion/ Deletion |
|---|---|---|---|---|
| 1 | M57764 M28453 | 587 | G replace A | Transition |
| 2 | M57764 M28453 | 588 | A replace G | Transition |
| 3 | M57764 M28453 | 611, 612, 613 | | Deletions |
| 4 | M57764 M28453 | 631 | G replace T | Transversion |
| 5 | M57764 M28453 | 669 | C replace G | Transversion |
| 6 | M57764 M28453 | 752 | T replace C | Transition |
| 7 | M57764 M28453 | 768 | G replace A | Transition |
| 8 | M57764 M28453 | 797 | C replace T | Transition |
| 9 | M57764 M28453 | 800 | C replace G, | Transversion |
| 10 | M57764 M28453 | 810 | T replace C | Transition |

## Discussions

On bovine GH expression level, Variation at 5' coding sequence has been previously studied (Sami *et al*, 1990; Wallis *et al*, 1995). It has been reported that signal peptide coding region and 5' untranslated sequence is involved in regulation and expression of bovine GH gene (Hecht and Geldermann, 1996). Hecht and Geldermann identified six sites of variable nucleotides in the 5' flanking region of GH gene and also identified one in the intron 1, so it is very important to study the possible polymorphisms or variations in the GH gene in local species of *Bubalus bubalis.*

RNA was converted into cDNA by RT-PCR and was amplified by using specific forward and reverse primers. The position of sequence that was amplified is considered important by a number of workers for polymorphism/variation in GH gene (Ferraz ALJ, 2003, 2006; Hediger *et al*, 1990; Carnicela *et al*, 2003; Chikuni, 1991; Lagziel and Soller, 1999; Unanian *et al*, 1994).

The variation exists due to the miss-splicing of the $4^{th}$ intron. It was investigated by Hampson *et al*, 1989 that $4^{th}$ intron containing bovine GH mRNA is found, which tells that this mRNA species is translated into a polypeptide. This may be due to that the splice donor and acceptor site of $4^{th}$ intron is not behaving and because of the absence of the appropriate sequence in exon (mutation of 13-15 amino acid), splicing event does not occur (Hampson *et al*, 1989). Ferraz *et al*, 2006 identified the deletions and insertions in the GH gene of the bovine. This research was done to find out the variants in the local species of *Bubalus bubalis* GH gene, which has not yet been reported.

The amplified part of GH gene was sequenced and it was revealed that the region has some variations from the original one. This is the confirmation of the work done by Ferraz *et al*, 2006. His aim was to identify and characterize polymorphisms within the gene of GHs; results were including the transitions, transversions and deletions.

In the present study GH gene was successfully amplified from placental tissues of buffalo and different variations in sequence of GH gene were also reported. These variations in different genes happen during the evolutionary process due to different factors. The level and number of variations may differ in different parts of words. The study of these variations is important to check the relatedness of organisms and they are also important in term of productivity of the animal especially in term of milk production and yield.

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