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Genotyping of Growth Hormone Gene in Egyptian Small Ruminant Breeds

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ABSTRACT
Small ruminants are considered as one of the major sources of meat and milk production in Egypt. Identification of the genes underlying livestock production traits leads to more efficient breeding programs and it is a promising way to improve production traits of farm animals. Growth hormone is a polypeptide hormone which is the major regulator of the metabolic procedures of growth and development and it is encoded by GH gene. In this study, we aimed to detect the genetic polymorphism of GH gene in major Egyptian sheep and goat breeds using PCR-RFLP and identify the single nucleotide polymorphism between different genotypes detected in these breeds. The primers used in this study flanked a 422 bp fragment from exons 2 and 3 of GH gene in sheep and goat. These PCR amplified fragments were digested with *Hae*III endonuclease and the results showed the presence of two genotypes; GG and AG with the absence of AA genotype in 149 tested animals for this gene. The total frequencies were 43.56 and 56.44% for GG and AG genotypes, respectively in 101 tested sheep animals whereas in goat animals, the total frequencies were 12.5 and 87.5% for GG and AG genotypes, respectively in 48 tested goat animals. The sequence analysis of purified PCR products representing these two detected genotypes declared the presence of a SNP (G→A) at position 55 in the amplified fragment which is responsible for the destruction of the restriction site GG^CC and consequently the presence of two different alleles G and A which were named in this work according to the detected SNP. The nucleotide sequences of sheep GH alleles G and A as well as goat GH alleles G and A were submitted to nucleotide sequences database NCBI/Bankit/GenBank and have accession numbers: KP120857, KP120858, KP120859 and KP120860, respectively. In conclusion, production improvements can be achieved by using new genetic technology for better selection of heritable traits through marker-assisted selection. Due to the reported association between genotype possess A/G nucleotide with different growth trait parameters, we recommend to increase this heterozygous genotype in native small ruminant breeds through the selection of animals have AG genotype of GH gene and enter them in breeding programs of Egyptian small ruminants as a way to increase their production traits.

Key words: Sheep, goat, GH, PCR-RFLP, SNPs

INTRODUCTION

In Egypt, there are several indigenous sheep and goat breeds. The most common sheep breeds include Barki, Rahmani and Ossimi while goat breeds include Baladi, Barki and Zaraibi (Galal *et al*., 2005). The contribution of both species to the total red meat produced in Egypt is about 9.1% (6.4% for sheep and 2.7% for goats). These goat and sheep breeds are reared using minimal resources, so they exposed to compromise and fluctuating environmental production challenges. Such indigenous genetic resources are thought to have acquired unique alleles and allelic combinations that could be important for animal productivity.

In animal industry, growth traits of animals are always of primary concern during breeding for its determinant economical value (Zhang *et al*., 2008). The growth hormone of mammals plays an important role in the control of reproduction through cell division, ovarian folliculogenesis, oogenesis and secretory activity (Gong, 2002; Hull and Harvey, 2002; Ola *et al*., 2008). Beside the reproduction, this hormone plays an important role in postnatal longitudinal growth and development, tissue growth, lactation, as well as protein, lipid and carbohydrate metabolism.

The growth traits are complex traits involving multiple genes, loci and interactions (Hua *et al*., 2009). Many candidate genes have been identified and selected for analysis based on a known relationship with productivity traits (Spelman and Bovenhius, 1998). In farm animals, promising candidate genes for many traits are in the growth hormone axis (Ishag *et al*., 2010).

Growth hormone is a peptide encoded by a single gene about 2.5 kb in length and consists of five exons and four intervening introns (Wickramaratne *et al*., 2010). It is produced by the somatotroph cells of the anterior lobe of the pituitary gland in a circadian and pulsatile manner (Ayuk and Sheppard, 2006). A lot of studies carried out in ruminants confirm a role of GH in regulation of mammary growth (Sejrsen *et al*., 2000; Akers, 2006) and show the effects of GH on growth in several tissues, including bone, muscle and adipose tissue (Hua *et al*., 2009). This study aimed to detect the genetic polymorphism in exons 2 and 3 of Egyptian sheep and goat GH gene and identify SNPs between different genotypes which were detected in these breeds.

MATERIALS AND METHODS

Blood samples and genomic DNA extraction: The whole blood samples were collected from 149 animals belonging to six native major small ruminant breeds; 45 from sheep Barki, 26 from sheep Rahmani, 30 from sheep Ossimi, 16 from goat Baladi, 15 from goat Barki and 17 from goat Zaraibi.

Genomic DNA was extracted from the whole blood according to the method described by Miller *et al*. (1988) with minor modifications. Briefly, blood samples were mixed with cold 2x sucrose-triton and centrifuged at 5000 rpm for 15 min at 4°C. The nuclear pellet was suspended in lysis buffer, sodium dodecyl sulfate and proteinase K and incubated overnight in a shaking water bath at 37°C. Nucleic acids were extracted with saturated NaCl solution. The DNA was picked up and washed in 70% ethanol. The DNA was dissolved in 1x TE buffer. DNA concentration was determined, using Nano Drop1000 Thermo Scientific spectrophotometer and then diluted to the working concentration of 50 ng μL^{-1} which is suitable for polymerase chain reaction.

Polymerase Chain Reaction (PCR): A PCR amplification reaction was performed using specific primer that was designed on the basis of DNA sequence of the GH gene (Accession: D00476) (Hua *et al*., 2009):

GH F: CTC TGC CTG CCC TGG ACT
GH R: GGA GAA GCA GAA GGC AAC

A PCR cocktail consisted of 1.0 µM of upper and lower primer, 0.2 mM dNTPs, 10x of PCR reaction buffer and 1.25 units of *Taq* polymerase (Fermentas). The cocktail was aliquot into PCR tubes with 100 ng of sheep or goat DNA. The reaction was run at 94°C for 5 min, 35 cycles of 95°C for 30 sec, touchdown annealing from 65-52°C for 30 sec, 72°C for 45 sec and a final extension at 72°C for 7 min. The PCR products were subjected to electrophoresis on 2% agarose gel stained with ethidium bromide to test the amplification success.

Restriction Fragment Length Polymorphism (RFLP): The PCR products were digested using restriction enzyme; *Hae*III (Fermentas). Ten microliter of PCR product was digested with 1 µL of FastDigest restriction enzyme for 5 min at 37°C. The restriction fragments were subjected to electrophoresis in 2% agarose ethidium bromide gel in 1x TBE buffer (0.09 M Tris-boric acid and 0.002 M EDTA). Gels were visualized under UV light and documented in FX Molecular Imager apparatus (BIO-RAD).

Sequencing analysis and single nucleotide polymorphism: The PCR products, representatives for each detected genotype of GH gene in different sheep and goat breeds were purified and sequenced by Macrogen Incorporation (Seoul, Korea). Sequence analysis and alignment were carried out using NCBI/BLAST/blastn suite to identify each single nucleotide substitution between different detected genotypes. Results of endonuclease restriction were carried out using FastPCR. The nucleotide sequence of each genotype for Egyptian sheep and goat GH gene were submitted to GenBank (NCBI, BankIt).

RESULTS AND DISCUSSION

Recently, genetic polymorphisms at candidate genes affecting economic traits have stimulated research interest because it is considered as an aid to genetic selection and to mark evolutionary relationships in different livestock breeds (Sodhi *et al*., 2007). Association of several polymorphic sites (SNPs) in different candidate genes with economic traits and their association with animal performance have been much investigated in different animal species such as cattle (Lucy *et al*., 1991; Schlee *et al*., 1994; Ge *et al*., 2003), sheep (Wallis *et al*., 1998; Bastos *et al*., 2001) and goats (Gupta *et al*., 2007).

Growth hormone gene, with its functional and positional potential, has been widely used for marker in several livestock species, including the cattle (Dybus, 2002; Ge *et al*., 2003; Beauchemin *et al*., 2006; Katoh *et al*., 2008), sheep (Marques *et al*., 2006) and goat (Malveiro *et al*., 2001; Boutinaud *et al*., 2003). This work aims to identify the RFLP and SNPs in Egyptian sheep and goat breeds.

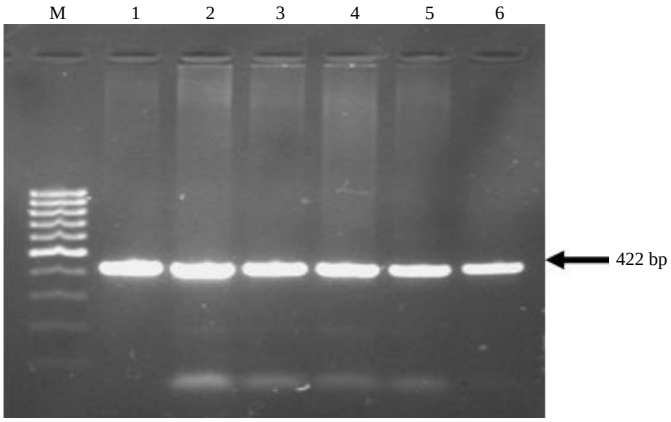

Fig. 1: Ethidium bromide-stained gel of PCR products representing amplification of GH gene in Egyptian sheep and goat animals. Lane M: 100 bp ladder marker, Lanes 1-6: 422 bp PCR products amplified from sheep and goat DNA

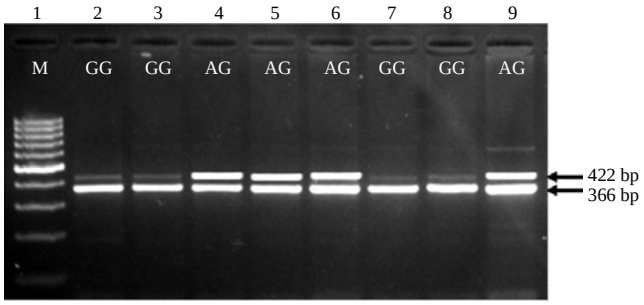

Fig. 2: Electrophoretic pattern obtained after digestion of PCR amplified fragment of GH gene from sheep and goat DNA with *Hae*III restriction enzyme. Lane 1: 100 bp ladder marker, Lanes 2, 3, 7 and 8: GG homozygous genotype with two digested fragments at 366 and 56 bp (Not showed in gel due to its small size). Lanes 4-6 and 9: AG heterozygous genotype with three digested fragments at 422, 366 and 56 bp (Not showed in gel due to its small size)

The primers used in this study flanked a 422 bp fragment from exons 2 and 3 of GH gene in sheep and goat. The amplified fragments obtained from all tested sheep and goat animals were at 422 bp (Fig. 1).

These PCR amplified fragments (422 bp) were digested with *Hae*III endonuclease. Depending on the presence or absence of the restriction site (GG^CC) at position 56^57, 3 different genotypes can be easily differentiated: GG with two digested fragments at 366 and 56 bp, AA with undigested one fragment at 422 bp and AG with three digested fragments at 422, 366 and 56 bp.

The results showed the presence of two genotypes; GG and AG with the absence of AA genotype in 149 tested animals for this gene (Fig. 2). The frequencies of GG and AG genotypes were 35.56 and 64.44% in sheep Barki animals (45 animals), 19.23 and 80.77% in sheep Rahmani animals (26 animals) and 76.67 and 23.33% in sheep Ossimi animals (30 animals), respectively with the total frequencies of 43.56 and 56.44% for GG and AG genotypes, respectively in 101 tested sheep animals for this gene. In tested goat animals, the frequencies of GG and AG genotypes were 0 and 100% for Baladi (16 animals), 13.33 and 86.67% for Barki (15 animals) and 23.53 and 76.47% for Zaraibi (17 animals), respectively with total frequencies of 12.5 and 87.5% for GG and AG genotypes, respectively in 48 tested goat animals for this gene.

These two detected genotypes are resulted from the presence of two different alleles; G (Fig. 3a) and A (Fig. 3b) in tested sheep and goat animals. The sequence analysis of the purified PCR products representing these two detecting genotypes GG and AG declared the presence of a single nucleotide polymorphism (G→A) at position 55 which is responsible for the destruction of restriction site GG^CC and consequently the differentiation between the two different genotypes G/G (Fig. 4a) and A/G (Fig. 4b) which were named in this work according to the detected SNP.

The nucleotide sequences of sheep GH alleles G and A as well as goat GH alleles G and A were submitted to nucleotide sequences database NCBI/Bankit/GenBank and have accession numbers KP120857, KP120858, KP120859 and KP120860, respectively.

(a)
CTCTGCCTGCCCTGGACTCAGGTGGTGGGCGCCTTCCCAG
CCATGTCCTTGTCC**G**GCCTGTTTGCCAACGCTGTGCTCCGG
GCTCAGCACCTGCATCAACTGGCTGCTGACACCTTCAAAG
AGTTTGTAAGCTCCCCAGAGATGTGTCCTAGAGGTGGGGA
GGCAGGAAGGGGTGAATCCGCACCCCCTCCACACAATGG
GAGGGAACTGAGGACCTCAGTGGTATTTTATCCAAGTAAG
GATGTGGTCAGGGGAGTAGAAATGGGGGTGTGTGGGGTG
GGGAGGGTTCCGAATAAGGCAGTGAGGGGAACCCCGCAC
CAGCTGAGACCTGGGTGGGTGTGTTCTCCCCCCAGGAGCG
CACCTACATCCCGGAGGGACAGAGATACTCCATCCAGAAC
ACCCAGGTTGCCTTCTGCTTCTCC

(b)
CTCTGCCTGCCCTGGACTCAGGTGGTGGGCGCCTTCCCAG
CCATGTCCTTGTCC**A**GCCTGTTTGCCAACGCTGTGCTCCGG
GCTCAGCACCTGCATCAACTGGCTGCTGACACCTTCAAAG
AGTTTGTAAGCTCCCCAGAGATGTGTCCTAGAGGTGGGGA
GGCAGGAAGGGGTGAATCTGCACCCCCTCCACACAACGG
GAGGGAACTGAGGACCTCAGTGGTATTTTATCCAAGTAAG
AATGTGGTCAGGGGAGTAGAAATGGGGGTGTGTGGGGTG
GGGAGGGTTCCGAATAAGGCAGTGAGGGGAACCACGCAC
CAGCTGAGACCTGGGTGGGTGTGTTCTCCCCCCAGGAGCG
CACCTACATCCCGGAGGGACAGAGATACTCCATCCAGAAC
ACCCAGGTTGCCTTCTGCTTCTCC

Fig. 3(a-b): Nucleotide sequence of (a) Allele G with nucleotide **G** at and (b) Allele **A** with nucleotide A at position 55

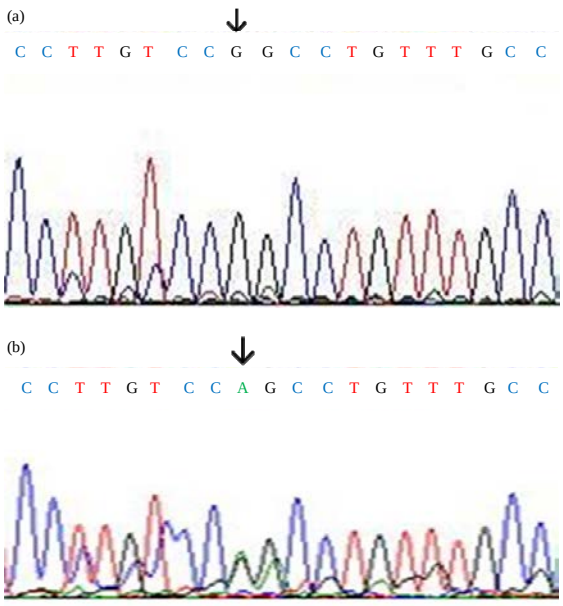

Fig. 4(a-b): Genotype (a) G/G and (b) A/G

The translation of the amplified segment from GH exons 2 (from nt 1-126) and exon 3 (from nt 354-422) using FastPCR declared the difference in amino acid (Gly→Ser) between G and A alleles (Fig. 5) according to G→A substitution at position 55 in the amplified fragment.

Allele G:
LCLPWTQVVGAFPAMSLS**G**LFANAVLRAQHLHQLAADTFKE
FERTYIPEGQRYSIQNTQVAFCFS

Allele A:
LCLPWTQVVGAFPAMSLS**S**LFANAVLRAQHLHQLAADTFKE
FERTYIPEGQRYSIQNTQVAFCFS

Fig. 5: Amino acid sequences of amplified fragments of GH from exons 2 and 3 for alleles G and A

DNA samples from different pure and crossed sheep breeds were digested with the restriction enzymes *Bam*HI, *Eco*RI and *Hind*III and screened for RFLP by Valinsky *et al*. (1990) using the bovine growth hormone gene as a probe. The results revealed that RFLP's was probably due to the presence of two alleles at the GH locus; a predominant allele (GH2), in which the growth hormone gene is duplicated and the two copies are located at a distance of 3.5 kb apart and a rare allele (GH1), in which the growth hormone gene is present as a single copy.

Hua *et al*. (2009) analyzed the polymorphism of GH gene as a genetic marker candidate for growth traits in Boer goat bucks. Two single nucleotide polymorphisms A781G and A1575G were identified by GH gene sequencing and PCR-RFLP analysis. AA genotype resulted in a significant decrease in birth chest girth ($p>0.03$) and weaning weight ($p>0.014$) comparing to AB genotype while CC genotype contributed to weaning height ($p>0.04$) greater than CD genotype. When in combination, AACD genotype was undesired for lower scores in a series of growth traits including body weight, length, height and chest girth at birth and weaning.

On the other hand, these two active mutations (A781G and A1575G) in growth hormone gene and their associations with litter size were investigated in both high and low prolificacy goat breeds by Zhang *et al*. (2011). Two genotypes in each mutation were detected in these two goat breeds. Neither BB nor DD homozygous genotypes were observed. Genotypic frequencies of AB and CC were significantly higher than those of AA and CD and dams with AB or CC genotypes had significantly larger litter sizes than those with AA and CD ($p<0.05$). On combining of the two loci, dams with ABCD genotype had the largest litter sizes when compared to the other genotypes ($p<0.05$). These results showed that these two loci of GH gene are highly associated with abundant prolificacy in goat breeds.

Wickramaratne *et al*. (2010) identified single nucleotide polymorphisms of GH gene and their association with growth traits in Osmanabadi and Sangamneri goat breeds. They revealed point mutations of G200T, A815G, A1753, C1763T and A1789G in GH gene sequence of both goat breeds. G200T resulted GG, GT and TT genotypes and of them, GT was associated with heavy body weight and GG with low body weight ($p\leq0.05$) in both the breeds. Similarly, AG of A815G revealed low body weight. AA of A1753 insertion revealed heavy body weight in both the breeds and 7% longer

body in Sangamneri breed. Heterozygote counterparts for C1763T and A1780G SNPs exhibited heavy body weights ($p \leq 0.05$). It was concluded that SNPs and their association with body weight may be useful in selecting goats for higher growth traits.

The present result agrees with the previous results obtained by Hua *et al*. (2009) and Zhang *et al*. (2011), where the presence of two genotypes; one of them is homozygous with G/G nucleotide and the other is heterozygous with A/G nucleotide in tested animals and the absence of homozygous genotype with A/A nucleotide and also the presence of heterozygous genotype with the highest frequencies in goat breeds. In Egyptian animals, the frequencies of AG heterozygous genotype were 56.44 and 87.5% for sheep and goat animals, respectively.

This genotype (A/G) is associated with high and best growth trait parameters like birth chest girth, weaning weight, body weight, length, height and large litter sizes. It may be due to the presence of both serine and glycine amino acids in the heterozygous animals and these amino acids are interconvertible; when the body needs any of them, it does not obtain it from the digestion of food, it uses glycine to produce serine and vice versa. Serine involved in the metabolic processes that burn glucose and fatty acids for energy and the body uses serine to make creatine which combines with water to "pump up" muscle mass. On the other hand, Glycine is required to build protein in the body. It is required for the synthesis of nucleic acids, the construction of RNA as well as DNA and synthesis of bile acids and other amino acids in the body and it helps in retarding degeneration of muscles (Lehninger *et al*., 2005).

CONCLUSION

In conclusion, production improvements can be achieved by using new genetic technology for better selection of heritable traits through marker-assisted selection. Due to the reported association between genotype possess A/G nucleotide with different growth trait parameters, we recommend to increase this heterozygous genotype in native small ruminant breeds through the selection of animals have AG genotype of GH gene and enter them in breeding programs of Egyptian small ruminants as a way to increase their production traits.

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