Polymorphisms in the exon-3 of leptin gene using tetra-primer ARMS-PCR in Egyptian buffalo

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ABSTRACT

The Egyptian buffalo is one of the Egyptian genetic resources which is irreplaceable and must be preserved, because of its great adaptability to local harsh environmental conditions and resistance to infectious diseases. A total number of 74 blood samples of female Egyptian buffalo were used to assess the allelic and genotypic frequency of G>A SNP at codon 159 of leptin gene. The tetra-primer amplification refractory mutation system-polymerase chain reaction (Tetra-primer ARMS-PCR), a single nucleotide polymorphism (SNP) technique allows for the simultaneous detection of alleles using a single PCR reaction and gel electrophoresis. Results revealed three genotypes GG, AG, and AA with frequencies of 0.49, 0.39, and 0.12, respectively. Allele frequencies of G and A were found to be 0.69 and 0.31, respectively. For the first time, allelic and genotypic frequency of G>A SNP at codon 159 in Egyptian buffalo was detected with the highest allelic frequency of allele A (0.31). Further studies are needed to provide more information about the association between this SNP and production and reproduction traits.

Key words: Egyptian buffalo, Tetra-primer ARMS-PCR, Leptin hormone.

INTRODUCTION

In Egypt, the Egyptian buffalo (*Bubalus bubalis*) is very significant because it is exceptional at adapting to challenging environmental conditions, resistant to infectious disease, and excellent at providing nutritional benefits. As a result of its flavor and composition, Egyptian consumers prefer buffalo milk (Abu El-Magd *et al.,* 2015). According to the Egyptian census of livestock, Egypt owns 3.8 million heads of buffalo, which represents about 37% of the total livestock, and buffalo milk production represents 70% of milk production in Egypt (Statistics of Ministry of Agriculture, 2018).

The recent advances in molecular genetic techniques during the past decades allow the scientists to achieve genetic studies at the molecular level. One of the applications of molecular genetic techniques is documenting and describing the genetic variation of DNA sequences between and within populations. A single nucleotide polymorphism (SNP) represents a single base variation in a DNA sequence at any given position, with an appearance rate of more than 1% in a given population. Compared with other genetic variations, SNPs spread across genomes with a very high frequency (Borodina *et al.,* 2004). Tetra-primer ARMS-PCR (Tetra-primer ARMS), a simple and inexpensive SNP genotyping technique, allows for the simultaneous detection of alleles using a single PCR reaction and gel electrophoresis. The technique only requires a set of four primers and does not require any expensive infrastructure or reagents (Ye *et al.,* 2001).

Leptin's primary transcript is 167 amino acids long, subsequently, the first 21 amino acid signal sequence is cleaved (Zhang *et al.,* 1994). Leptin is primarily produced in adipose tissue and secreted into the bloodstream (Deshpande *et al.,* 2014). Its expression also occurs in other cells and tissues, including the placenta (Hoggard *et al.,*1997), mammary glands (Smith-Kirwin *et al.,* 1998), skeletal muscles (Wang *et al.,*1998), and pituitary glands (Morash *et al.,*1999), in which LEP acts as autocrine or paracrine. There are very different functions of the LEP hormone, including modulation of reproduction (Yu *et al.,* 1997), metabolism (Agarwal *et al.,* 2009), mammary gland development, as well as cell differentiation and proliferation (Onneta *et al.,* 2002). The leptin gene has been mapped to chromosome 8 (BBU 8q32) in buffaloes (Vallinoto *et al.,* 2004), and consists of three exons, of which the first exon is not translated into protein.

In 2007, Orrù *et al.* sequenced the leptin coding region (exon2 and exon3) on a panel of 32 Italian River Buffalo and two Egyptian River Buffalo. Twelve new SNPs were detected. One of the new SNPs, G>A in exon 3, causes a change in the second position of

codon 159, resulting in arginine (R) to glutamine (Q) substitution of the primary transcript (138 in mature peptide). However, this polymorphism was found in the heterozygous state in only one sample of Italian buffalo, whereas the other animals showed a GG homozygote genotype (Orrù *et al.,* 2007). The existence of an A allele was confirmed by DNA sequencing of 390 Murrah and Philippine Carabao buffaloes, with an allele frequency of 0.18 (Seong and Kong, 2012). Also, Kale *et al.,* 2013 detected the same variation by SSCP in Murrah, Surti and Bhadawari, with a high allele frequency of 0.3. There is no study about the genotypic frequency of the A > G SNP at codon 159 in Egyptian buffalo. Therefore, the aim of the present studies was to assess the allelic and genotypic frequency of SNP A > G at codon 159 in Egyptian buffalo by Tetra-primer ARMS-PCR.

MATERIALS AND METHODS

DNA samples

A total of 74 blood samples were collected from Egyptian buffalo. The samples were randomly taken from the Agricultural Experiments Station (AES) which belongs to the Faculty of Agriculture, Cairo University, Giza, Egypt. Approximately 5 ml of blood per animal was obtained by jugular venipuncture in a K3-EDTA tube containing anticoagulant. The genomic DNA was extracted from whole blood using a commercial kit according to the manufacturer's protocol for ISOLATE II Genomic DNA Kit, Bioline, Cat No. BIO-52066.

Genotyping

The Tetra-primer ARMS-PCR and primers for G>A SNPs at codon 159 were designed by the freely available online program http://primer1.soton.ac.uk/primer1.html The list of primer sequences annealing temperatures, and the sizes expected of PCR products are given in Table 1. In a final volume of 20µl, a PCR reaction containing 1µl genomic DNA, 2µl each primer, 0.5µl $MgCl_2$, 10 µl GoTaq® Green Master Mix (Promega, Madison, USA), and nuclease-free water up to 20µl were performed. The PCR program included a 5-minute denaturing step at 95°C, 35 cycles of [Denaturation 95°C for 30 Sec, Annealing 64°C for 30 Sec, Extension 72°C for 1-minute] and a final 7-minute extension step at 72 °C.

Statistical analysis

Allelic and genotypic frequencies were calculated by simple counting. Hardy-Weinberg equilibrium was tested by gene-calc web http://gene-calc.pl/hardy-weinberg-page.

RESULTS AND DISCUSSION

The tetra-primers were designed for the detection of the G>A point mutation at codon 159 of the leptin gene in Egyptian buffalo. Three genotypes are shown in Fig. 1 for GG, AG, and AA with frequencies of 0.49, 0.39, and 0.12, respectively. Allele frequencies of G and A were found to be 0.69 and 0.31, respectively. The accordance of the mentioned SNPs with HWE was authorized by the gene-Calc web at 0.05 of the level of significance.

The allelic and genotypic frequency of the amino acid substitution arginine (R) to glutamine (Q) at position 159 was detected in buffaloes for the first time by Orrù *et al.,* (2007) with an allelic frequency of 0.01, whereas the heterozygote GA genotype was not detected. The higher frequency (0.18) was detected by Seong and Kong (2012) in Murrah and Philippine Carabao buffaloes, with a low frequency of homozygote genotype AA (0.03). The highest allelic frequency of allele A (0.3) was detected by Kale *et al.,* (2013), and the same results were reported in the present study. The Single-Strand Conformation Polymorphism (SSCP) for leptin exon 3 of Egyptian buffalo failed to detect the G > A point mutation at codon 159 (Ghoneim *et al.,* 2016), whereas the tetra-primer (ARMS-PCR) in our study was declared for fast and efficient detection of the G > A SNP at codon 159 in the leptin gene.

The R159Q substitution is located at position 138 in the C-terminal region of the mature peptide, within helix D (Reicher *et al.,* 2011). Arginine is a polar, positively charged (basic amino acid) and has an uncharged R group, while glutamine is polar, uncharged and has an amide R group. The expected effect of the R159Q SNPs on protein functions was studied by Mahrous *et al.,* (2020). The R159Q lowered the stability of the mature leptin peptide tertiary structure by 0.25 kcal/mol and classified this mutation as a neutral mutation with a total PredictSNP expected accuracy of 83%.

CONCLUSION

In this study, for the first time, allelic genotypic frequencies of G>A SNP at codon 159 in Egyptian buffalo were detected, with

the highest allelic frequency of allele A (0.31). Further studies are needed to provide more information about the association between this SNP,production and reproduction traits.

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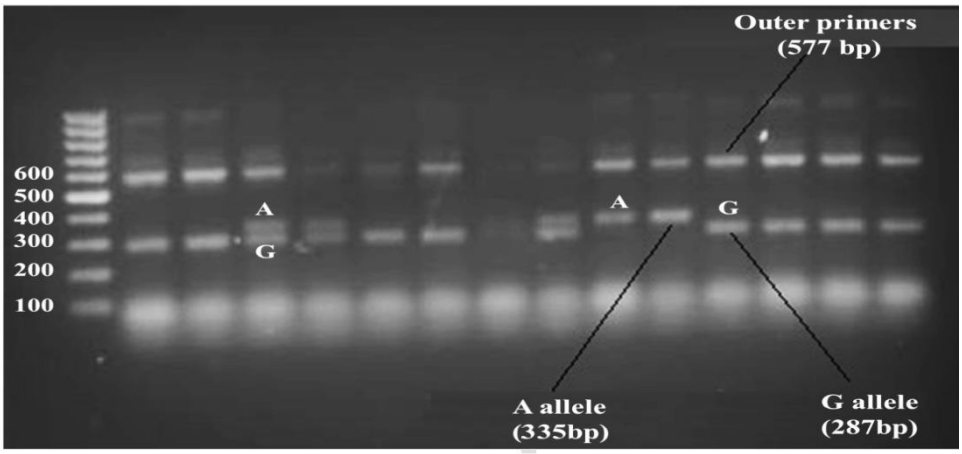

Figure 1: The amplified products by Tetra-Primer ARMS–PCR on agarose gel

Table 1: Nucleotide sequences of primers, Annealing temperature, and the sizes expected of PCR products.

Primer	Primer sequence	Annealing temperature
LEP inner-F (G allele)	GGGTCACTACAGGACATGTTTC**G**	64 C
LEP inner-R (A allele)	CCAGGACTGAGGTCCAGCTTC**T**	
LEP outer-F	CAGAGGGTCACTGGTTTGGACTT	
LEP outer-R	CTCCGGTCTACTGTTTGCTGGAT	
Product size for G allele: 287		
Product size for A allele: 335		
Product size of two outer primers: 577		

Allele specificity is conferred by A second mismatches T instead of G and T instead of C (demonstrated by underlined letter) are introduced at the third position from the 3′ end of each of the Forward and Reverse inner primers.

تعدد الأشكال في إكسون ٣ لجين اللبتين باستخدام Tetra-primer ARMS-PCR في الجاموس المصري

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الملخص العربي

يحظى الجاموس المصري بأهمية كبيرة في مصر، لما له من قدرة كبيرة على التكيف مع الظروف البيئية القاسية، ومقاومة الأمراض المعدية، كما يفضل المستهلكون في مصر لبن الجاموس لما له من مذاق مميز ومكونات جيدة. لهرمون اللبتين وظائف كثيرة ومتعددة، بما في ذلك التأثير على التكاثر، والتمثيل الغذائي، وتطور الغدة اللبنية، وكذلك تمايز الخلايا وتكاثرها ويتم إنتاج هرمون اللبتين بصورة أساسية في الأنسجة الدهنية ويتم التعبير عنه أيضًا في بعض الأنسجة الأخرى، بما في ذلك المشيمة والغدد اللبنية والعضلات الهيكلية والغدد النخامية. Tetra-primer ARMS-PCR (Tetra-primer ARMS) هي تقنية بسيطة وغير مكلفة لدراسة الاختلافات الوراثية، حيث تساعد هذه الطريقة فى الكشف المتزامن للأليلات باستخدام تفاعل PCR واحد وتفريد كهربي للجيل. لذلك، كان الهدف من هذه الدراسة هو تقدير تكرار الأليل وتكرار التركيب الوراثى لـ G>A SNP في الكودون رقم ١٥٩ لجين اللبتين في الجاموس المصري حيث تم جمع ٧٤ عينة دم من إناث الجاموس المصرى ومن خلالها أمكن الكشف عن ثلاثة طرز وراثية هى GG و AG و AAبتكرارات ٠,٤٩ و ٠,٣٩ و ٠,١٢ على الترتيب، وكان تكرار الأليل G و 0,69 A و ٠,٣١ على الترتيب. لأول مرة، تم تقدير تكرار الأليل وتكرار التركيب الوراثية للاختلاف الوراثى G>A للكودون ١٥٩ في الجاموس المصري ، مع أعلى تكرار أليلي للأليل .(0.31) A هناك حاجة إلى مزيد من الدراسات لتوفير مزيد من المعلومات حول العلاقة بين هذا الاختلاف الوراثى وصفات الإنتاج والتكاثر.

الكلمات الاسترشادية: الاختلافات الوراثية، هرمون اللبتين، الجاموس المصرى.