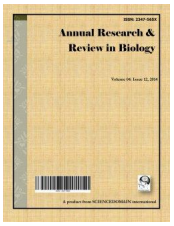

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Study on Genetic Polymorphism of IGFBP-3 Gene in Egyptian Buffalo

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Authors' contributions

This work was carried out in collaboration between all authors. Author OEO designed the study, performed the data analysis, wrote the protocol and wrote the first draft of the manuscript. Author AAE performed the practical work and followed the publication process. Author AEED managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present work was carried out to study the genetic characteristics of the IGFBP-3 gene in buffaloes reared in Egypt, where it is considered as one of the important molecular markers for productivity traits like growth and immunity in livestock species. One-hundred animals were used in this research work.
Methods: The studied gene was amplified through polymerase chain reaction technique. Afterwards, the amplified fragment at 651-bp was digested with three different endonucleases; *Hae*III, *Msp*I, and *Taq*I. The genetic character of the IGFBP-3 gene was studied by using PCR-RFLP and nucleotide sequencing.
Results: The PCR products after the digestion with those restriction enzymes revealed that the presence of the following fragments: two fragments at 506- and 145-bp with *Msp*I two fragments at 240- and 411-bp with *Taq*I; and eight fragments at 199-, 164-, 154-, 56-, 36-, 18-, 16- and 8-bp with *Hae*III. The restriction digestion of the amplified fragments of the IGFBP-3 gene did not show a

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genetic polymorphism or nucleotide substitution where all restricted fragments yielded from the digestion with three restriction enzymes were of the same sizes.
Conclusion: Our findings indicated that the absence of the genetic polymorphism of the IGFBP-3 gene in Egyptian buffalo. Based on our results in addition to the significant effect of this gene on different productivity traits, the crossing between Egyptian buffalo with other breeds, particularly the Italian breed, is needed for more improvements of Egyptian buffalo's productivity where the Italian buffaloes characterized by high growth and fertility phenomena.

Keywords: PCR; RFLP; Egyptian buffalo; IGFBP-3.

1. INTRODUCTION

Insulin-like growth factors (IGFs) IGF-I, IGF-II and their binding proteins (IGFBPs) exist in a widespread of tissues throughout the body where they regulate the anabolic and catabolic pathways, which hold an essential part in the body growth [1]. Regarding IGFBPs, they are a group of proteins containing at least six analogous proteins that bind IGFs and regulate many of their biological activities [2]. The IGFBP-3 gene is one of them, which is considered a promising molecular marker which has different roles in production traits like growth, reproduction, and immunity [3]. Referable to the important function of the IGFBP-3 in different biological activities of animals, the polymorphism in IGFBP-3 affects on in different livestock [4,5,6].

The IGFBP-3 gene in bovine is situated on chromosome number 4 [7] and has mRNA where its length is 1.65 kb [8]. The molecular study of this gene declared that the total length of it is 8.9 kb [9]. Many authors studied the nucleotide sequences of the IGFBP-3 gene in bovine [1,10,11]. The polymorphic characters and nucleotide sequence of the IGFBP-3 gene were reported in cattle by Maciulla et al. [12]; Haegeman et al. [13]; Sun et al. [11] and Othman et al. [2]. Further, the previous studies were also studied in the buffalo [5,14], sheep [15] and the goat [16,17]. In the same context, several authors recorded a relationship between the IGFBP-3 genotypes with production characteristics in cattle [2,18]. Cheong et al. [19] revealed that the polymorphism in the promoter region (-854G>C) is associated with carcass trait parameters at p= 0.03. Further, in the goat, the effect of the IGFBP-3 polymorphism on fiber traits including fiber length, hair length, and cashmere weight [20] and its effect on the litter size [17] was reported. Regarding the immune function of this gene, Choudhary et al. [21] identified the effect of its genotype on serum IgG level of calves at a significant level ($p<0.05$).

The Egyptian buffalo plays an important task in the Egyptian economy, where it is the major supply of meat and milk. The productivity improvements of these foodstuffs are economically needed to face the overpopulation in Egypt. One of the improved ways of buffalo's productivity is the genetic improvement depending on the molecular markers. Therefore, the present work aimed to genetically characterize the IGFBP-3 gene in buffaloes reared in Egypt using PCR-RFLP and nucleotide sequencing analysis.

2. MATERIALS AND METHODS

2.1 Ethics Statement

The blood samples used in this study were collected by veterinarians during routine blood sampling on commercial farm animals (for medical care or follow up). These animals were not linked to any experimental design, and the blood sampling was not performed specifically for this study. All the samples and data processed in this study were obtained with the breeders and breeding organizations' consent.

2.2 Animals and DNA Extraction

The blood samples were collected from one hundred buffaloes. Genomic DNA was extracted from the whole blood according to the method described by Miller et al. [22] 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, which is used in polymerase chain reaction.

2.3 Polymerase Chain Reaction (PCR)

The DNA fragment of the studied gene was amplified by using the polymerase chain reaction technique according to Mullis et al. [23]. This amplified fragment covered a part of exon 2, intron 2, exon 3 and a part of intron 3. A PCR cocktail consists of 1.0 µM upper and lower primers [12] specific for tested gene, 0.2 mM dNTPs (Fermentas**,** Thermo Fisher Scientific Inc.), 10 mM Tris (pH 9), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin (w/v), 0.1% Triton X-100 and 1.25 units of *Taq* polymerase (Fermentas**,** Thermo Fisher Scientific Inc.). The cocktail was aliquoted into PCR tubes with 100 ng of buffalo DNA. The reaction was cycled with the following conditions; initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation at 94°C, annealing at 60°C and extension at 72°C, each step for 1 min and the final extension for 5 min at 72°C. The amplification was verified by electrophoresis on 2% agarose gel (w/v) in 1x TBE buffer using GeneRuler^{TM} 100-bp ladder. The gel was stained with ethidium bromide and visualized on UV trans-illuminator.

Forward primer: 5′-CCA AGC GTG AGA CAG AAT AC -3′

Reverse primer: 5′-AGGAGG GAT AGG AGC AAG AT-3′

2.4 Restriction Fragment Length Polymorphism (RFLP)

The PCR products were digested using three different restriction enzymes; *Hae*III, *Taq*I and *Msp*I (Fermentas, Thermo Fisher Scientific Inc.). 10 µl of PCR product were digested with 1 ul of FastDigest restriction enzymes for 5 min at 37°C for *Hae*III and *Msp*I restriction endonucleases and at 65°C for *Taq*I enzyme. The restriction fragments were subjected to electrophoresis in 2% agarose/ethidium bromide gel (GIBCO, BRL, England) in 1× TBE buffer (0.09 M Tris-boric acid and 0.002 M EDTA). Gels were visualised under UV light and documented in FX Molecular Imager apparatus (BIO-RAD).

2.5 Sequence Analysis

The PCR products for each genotype of the tested gene were purified and sequenced by Macrogen Incorporation (Seoul, Korea). Sequence analysis and alignment were carried out using NCBI/BLAST/blastn suite. Results of endonuclease restriction were carried out using FastPCR.

3. RESULTS AND DISCUSSION

Buffaloes are considered the major source of meat in Egypt and due to over populations; the increasing of meat production is progressively needed to overcome the gap between the consumption and supplies of this important foodstuff. Insulin-like growth factor binding protein-3 is a protein belonging to the large family of IGFBPs. These family members involved in many cellular functions, including growth, immunity, and metabolism [19]. The animal production suffers a huge loss in milk and meat due to the infection of livestock with different diseases. So, the raising of animals' disease resistance can help in the improvement of animal production. The selection of buffalo having better growth and immunity performances helps in the genetic improvement of buffaloes' populations using marker-assisted selection [24,21].

IGFBP-3 plays an important role in development, growth, reproduction [25,26] and in immune function of the animals [27]. The identification of genetic polymorphism and nucleotide structure of the IGFBP-3 gene was reported in different livestock, including cattle [28], buffalo [5] and sheep [29].

The current work aimed to detect the genetic structure and polymorphism of Egyptian buffalo IGFBP-3 gene by using PCR-RFLP and nucleotide sequencing. The fragment which was amplified using PCR composed of 651-bp extended from exon 2 to intron 3 of Egyptian buffalo IGFBP-3 gene (Fig. 1).

In this study, three restriction enzymes were used to identify the polymorphism among the 651-bp amplified fragment of buffalo IGFBP-3 gene. The used endonucleases have different restriction sites; *Hae*III (**GG^CC**), *Msp*I (**C^CGG**) and *Taq*I (**T^CGA**). The digestion of the PCR products with these three restriction enzymes revealed the presence of two fragments at 506- and 145-bp with *Msp*I, two fragments at 411- and

240-with *Taq*I and eight fragments at 199-, 164-, 154-, 56-, 36-, 18-, 16- and 8-bp with *Hae*III (Figs. 2 and 3).

The restriction digestion of the amplified fragments of the IGFBP-3 gene did not show any genetic polymorphism or nucleotide substitution among the tested buffaloes, where all restricted fragments yielded from the digestion with three restriction enzymes had the same sizes (Fig. 2). The present result confirmed the finding by Padma et al. [5] who examined the presence of genetic polymorphism among the IGFBP-3 gene in four river buffalo breeds; Murrah, Surti, Jaffarabadi and Nagpuri breeds in India. Using the same primers and the restriction enzymes, they reported the IGFBP-3 monomorphism in all tested buffaloes belonging to these four Indian riverine buffalo breeds.

This monomorphic genetic pattern of the IGFBP-3 gene in Egyptian buffalo is different from the polymorphic pattern of this gene in Egyptian cattle [2]. The restriction digestion of Egyptian cattle IGFBP-3 gene with *Hae*III endonuclease

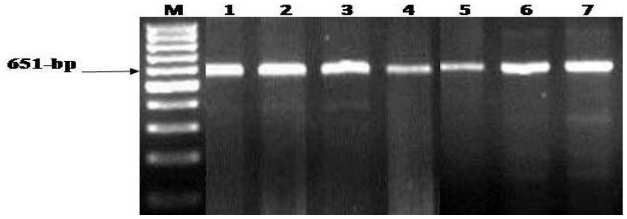

Fig. 1. PCR products of Egyptian buffalo IGFBP-3 gene
Lane M: 100-bp DNA ladder marker.
Lanes 1-7: 651-bp PCR products amplified from Egyptian buffalo DNA.

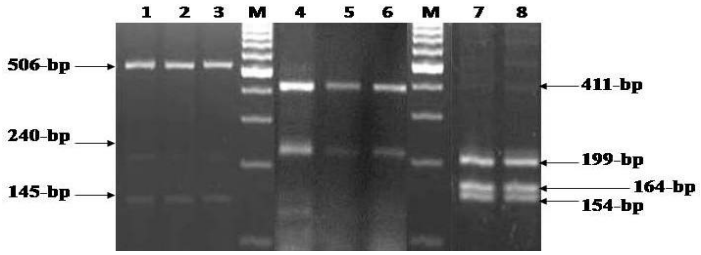

Fig. 2. Restriction patterns of Egyptian buffalo IGFBP-3 gene
Lanes M: 100-bp molecular markers
Lanes 1-3: MspI restriction pattern with two fragments at 506- and 145-bp
Lanes 4-6: TaqI restriction pattern with two fragments at 411- and 240-bp
Lanes 7-8: HaeIII restriction pattern with 8 fragments at 199-, 164-, 154-, 56-, 36-, 18-, 16- and 8-bp (small fragments less that 100-bp did not appear in the figure).

CCAAGCGTGAGACAGAATACGTGAGAGCTTTTCCTCTTGCTGATGTGGGGGTGG**GG^CC**ACCT**G**
G^CCTGGGTATCCAGAGATCACAGGGTCACCATTACTCAAGAGCCCAGCAGTTACTCCAGTGGT
CCTGCTGATGCACCAAGCAGCTGCAAGCCCTTCCTTACAGAAGGGATATTGACCCTCCCCTATG
GCAGAGATCCCAGGAGAATCAGTGCACTGCTCTCCA**GG^CC**TCGGCTGGGCAGAGCAGTGTTCT
CACAAAGCT**GG^CC**TCTTTTTGTCACTT**GG^CC**TCTGAGTGTCCT**GG^CC**TGTGTGTCCCTGTCC
CAGTCCTGTAGCTTGCCCTGGGGAATCACAAGAGAGACAGGGCTGTGGTTGGCATCTGCACAG
GAACGGTGACAACTAAATCAGACAAAAGATAC**T^CGA**GGAGCACGTGGTCAGTTCCCTGGGCGT
CACAGGGTTTTATCAGACACAGAGTTCCCAGGTAACCCGTGCCTCCTCCCCAGG**GG^CC**CTGCC
G**C^CGG**GAAATGGAAGACACGCTGAACCACCTCAAGTTCCTGAACATGCTCAGCCCCAGGGGCA
TCCACATTCCCAACTGCGACAAGAAGGGCTTCTACAAGAAAAAGCAGGTGAGCACCATCCAAGC
ATCTTGCTCCTATCCCTCCT

Fig. 3. The restriction sites of the three used endonucleases
C^CGG for *Msp*I, T^CGA for *Taq*I and GG^CC for *Hae*III

Fig. 4. Single nucleotide substitution C (buffalo) → A (cattle) in IGFBP-3 gene

showed a nucleotide substitution (C→A) at position 299 of the 651-bp amplified fragment of this gene. This nucleotide substitution is absent among Egyptian buffalos (Fig. 4).

Choudhary et al. [21] examined the association of IGFBP-3 polymorphism with immune functions in Holstein Friesian×Hariana crossed breed. They found that *Hae*III-AB genotype had significantly higher serum IgG level than the *Hae*III-AA. This finding indicated the role of IGFBP-3 gene on serum IgG level and immune functions of cattle calves. The finding by Choudhary et al. [18] revealed a significant effect of IGFBP-3 genotypes on birth weight and body weight in this crossed breed. In this context, animals with the AB genotype exhibited higher properties such as birth weight and body weight other than the animals having AA genotype. Furthermore, the promising effect of the IGFBP3 gene as a candidate gene for a growth trait was investigated in cattle by Cheong et al. [19]. They examined the association between IGFBP3 polymorphisms and cold carcass weight and the marbling score among Korean native cattle. Statistical analysis revealed that one polymorphism in the promoter region showed putative associations with marbling score.

In the goat, the effect of the IGFBP-3 polymorphism on fiber traits in Chinese Inner Mongolian cashmere goats was investigated by Liu et al. [20]. In this context, statistical analysis by least squares method showed that a significant effect of genotypes on cashmere properties like fiber length, hair length, and cashmere weight. Further, results revealed that the studied animals of AB and BB genotypes exhibited higher cashmere properties such as weight, fiber length, and hair length other than animals possessing AA genotype. Lan et al. [17] found that the presence of four SNPs among the IGFBP-3 gene and their association with litter size and weight traits in four Chinese goat breeds. Moreover, the frequencies of polymorphism in different breeds showed significant differences in wool, dairy and meat types. In the same context, the attained data showed that the IGFBP-3 genotype was strongly related to the litter size. This observation highlighted the importance of the IGFBP-3 gene as a promising gene for a reproduction trait in goat.

4. CONCLUSION

As a conclusion, due to the absence of the IGFBP-3 polymorphism in Egyptian buffalo and the important effect of genetic polymorphism of this gene on different productivity traits, the crossing between Egyptian buffalo with other breeds, especially Italian breed is needed for more improvements of Egyptian buffalo's productivity where the Italian buffaloes are characterized by high growth and fertility phenomena.

CONSENT AND ETHICAL APPROVAL

The blood samples used in this study were collected by veterinarians during routine blood sampling on commercial farm animals (for medical care or follow up). These animals were not linked to any experimental design, and the blood sampling was not performed specifically for this study. All the samples and data processed in this study were obtained with the breeders and breeding organizations' consent.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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