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Genetic Polymorphism of *Cyp19* Gene and its Association with Ovarian Activity in Egyptian Buffaloes

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Abstract: One of the physiological causes for true anestrum is a low level of ovarian estrogens. Estrogens are imperative for reproductive development, fertility, bone growth and sexual behavior. The key enzyme in estrogen biosynthesis is cytochrome P450 aromatase, the protein product of *CYP19* gene. The genetic polymorphism of *CYP19* gene might influence fertility performance in cattle and buffalo. This study aimed to identify the genetic polymorphism of *CYP19* gene and its association with ovarian activity in Egyptian buffaloes using PCR-SSCP and sequencing analysis. Blood samples were collected with or without EDTA from female Egyptian buffaloes. Gynecological examinations (aided by Ultrasonography) were carried out twice for two successive weeks. Animals which did not show estrous signs during the breeding season (September - May) and have small non functioning ovaries were considered to suffer from ovarian inactivity. The syndrome was confirmed by assaying progesterone in serum. The genomic DNA was extracted from the whole blood to investigate the genetic polymorphism in *CYP19* gene. Results revealed high prevalence of ovarian inactivity in Egyptian buffalo (41.54%) with higher incidence in heifers (59.23%) than in cows (39.04%). SSCP analysis of 351-bp fragments covered exon 2 of Egyptian buffalo *CYP19* gene showed the presence of three different genotyping patterns in the thirty tested buffaloes. The frequencies of these patterns were 46.67%, 40% and 13.3% for patterns 1 (T/T), 2 (C/C) and 3 (T/C), respectively in normal cyclic animals group whereas these frequencies were 13.33%, 73.33% and 13.33%, respectively in acyclic animals group. PCR products of different genotyping patterns for *CYP19* genes were purified and sequenced. The nucleotide sequences of two different alleles C and T of *CYP19* gene in Egyptian buffalo were submitted to GenBank (NCBI, BankIt) and have the accession nos. KJ551928 and KJ551929, respectively. Our sequence finding indicates that the substitution of nucleotide T by C at position 72 in the amplified fragments is related to the ovarian activity in Egyptian buffalo where these two nucleotides are present at high frequencies in normal cyclic and acyclic animals, respectively whereas the heterozygote TC is present with the same frequency in the two tested groups.

Key words: CYP 19 gene · *Folliculo genesis* · Ovarian inactivity · Buffalo cows

INTRODUCTION

Reproductive efficiency has a high priority in all breeding systems, especially in seasonal breeder animals. Buffalo are capable of breeding throughout the year, but in many countries like Egypt, the seasonal trend of ovarian activity, makes the opportunity of buffalo get pregnant is time limited [1]. Comparing to cattle, buffalo ovaries are smaller and contain fewer primordial follicles (10,000 - 20,000) [2] as compared with over 100,000 in cattle [3].

The signs of estrus in buffalo are less overt than in cattle whereas the peak concentrations of progesterone and oestradiol-17â are less. Field surveys on reproductive disorders revealed that anestrum was the most common cause of infertility in buffaloes particularly in Egypt [4-5].

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One of the physiological causes for true anestrum is a low level of ovarian estrogens [6] which are the main imperative for reproductive development, fertility, bone growth and sexual behavior.

Genomics screening of folliculogenesis may help to improve the reproductive performance of Egyptian buffalo. The key enzyme in estrogen biosynthesis is cytochrome P450 aromatase, the protein product of *CYP19* gene. The mutations in *CYP19* gene causes androgen excess and decrease of estrogen which may lead to ovarian inactivity and deficient follicular development [7].

The genetic polymorphism of *CYP19* gene might influence fertility performance of cattle [8] and Indian Murrah buffalo [7]. Genetic characterization of *CYP19* gene and its allelic variants in animals with different levels of fertility performances have not been studied in Egyptian buffaloes, so this study aimed to identify the genetic polymorphism of *CYP19* gene and its association with ovarian activity in Egyptian buffaloes using PCR-SSCP and sequencing analysis.

MATERIALS AND METHODS

This investigation was carried on a total number of 2723 buffaloes (2382 buffalo cows and 341 heifers) at Lower Egypt. These animals were raised in small holder farms at approximately the same management conditions. A full case history and owner complaint of each animal were recorded. The general health condition was examined and the body condition score was recorded. Gynecological examinations were carried out twice for two successive weeks at least to register the reproductive status and/or disorder. Examination was aided by Ultrasonography (PiaMedical Flacse Saote, Netherland) with an endorectal array of 8.6 M Hertz. Animals which did not show estrous signs during the breeding season (September - May) and have small non functioning ovaries were considered to suffer from ovarian inactivity.

Blood Sampling: Blood samples were collected with or without EDTA from female Egyptian buffaloes. Serum was separated from coagulated blood samples by centrifugation (3000xg, 15 minutes) and kept at -20°C until use for assaying of progesterone level in serum. DNA extraction was done from whole blood and kept in -20°C for genomic investigation.

Assaying of Progesterone Level: Serum progesterone level was assayed using ELIZA microwell technique using kits from DIMA (Germany). The kit had a sensitivity of 2.0 pg/ml with inter- and intra-run precision coefficient of variations of 2.9 and 4.85, respectively.

Genomic Dna Extraction: Genomic DNA was extracted from the whole blood according to the method described by Miller *et al*. [9] 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 50ng/µl which is suitable for polymerase chain reaction.

Polymerase Chain Reaction (PCR): The DNA fragment of the *CYP19* gene was amplified through polymerase chain reaction technique developed by Mullis *et al*. [10]. This amplified fragment covered a part of exon 2 of this gene. A PCR cocktail consists of 1.0 μM upper and lower primers [7], 0.2 mM dNTPs and 1.25U of Taq polymerase. The cocktail was aliquot 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™ 100-bp ladder as a molecular weight marker for confirmation of the PCR product lengths. The gel was stained with ethidium bromide (1 µg/µl) and visualized on UV trans-illuminator.

Forward primer: 5′-GGG CTT GCT TGT TTT GAC TC-3′
Reverse primer: 5′-CTG GTA TTG AGG ATG TGT CC-3′

Single Strand Conformation Polymorphism (SSCP): SSCP technique was used to identify the genetic polymorphism of *CYP19* gene in Egyptian buffalo. PCR products were resolved by SSCP analysis according to the method of Orita *et al.* [11]. PCR product was diluted in denaturing solution, denatured at 94°C for 5 min, chilled on ice and resolved on polyacrylamide

(12%, Acrylamide:bisacrylamide 49:1) with 5% glycerol. The electrophoresis was carried out in a vertical unit in 1x TBE buffer at 200 V and 20 mA for 5 h at 4°C, the gel was stained with silver staining [12] then visualized on light box and photographed by digital camera.

Sequence Analysis: The PCR products of different genotyping patterns for *CYP19* genes were purified and sequenced by Macrogen Incorporation (Seoul, South Korea) to identify the SNPs between these different genotyping patterns. Sequence analysis and alignment were carried out using NCBI/BLAST/blastn suite. The nucleotide sequences of two different alleles for *CYP19* gene in Egyptian buffalo were submitted to GenBank (NCBI, BankIt).

RESULTS

Ovarian Inactivity: Rectal examination, confirmed by ultrasonography, revealed that out of the total examined animals, 41.53% showed ovarian inactivity. These animals had small sized uteruses and small hard ovaries in texture with no physiological structures on their surface. Moreover, these animals showed no signs of heat after calving since at least 6 months during the breeding season. The incidence was higher in heifers (59.23%) than in cows (39.04%). Animals showed ovarian inactivity are most likely have poor body condition score (2.08±0.11) versus (2.88±0.28) for cyclic group.

Serum Progesterone Level: Serum progesterone level was very low or even not detectable in acyclic animals if compared to normal cyclic animals either in follicular or luteal phase of the estrous cycle as shown in Table 1.

Genotyping of Cyp19 Gene: Thirty Egyptian buffaloes were subjected for PCR-SSCP to detect the different genotyping patterns of *CYP19* gene. These animals were divided into two equal groups according to the ovarian maturation performance; normal cyclic and acyclic animals.

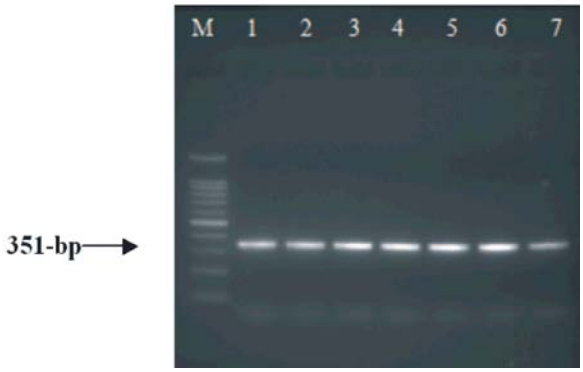

Fig. 1: Ethidium bromide-stained gel of PCR products representing amplification of *CYP19* gene in Egyptian buffalo.
Lane M: 100-bp ladder marker.
Lanes 1-7: 351-bp PCR products amplified from Egyptian buffalo DNA

Table 1: Serum progesterone level (ng/ml) in buffalo-cows having inactive ovaries (Mean±SE)

	Normal cyclic animals	Acyclic animals
Follicular phase	0.50±0.11	≤ 0.02
Luteal phase	2.94±0.36	

The primers used in this study flanked a 351-bp fragment covered exon 2 of Egyptian buffalo *CYP19* gene. The amplified fragments obtained from all thirty tested buffalo DNA were at the expected size (351-bp) as showed in Fig. 1.

SSCP analysis showed the presence of three different genotyping patterns in the thirty tested buffaloes (Fig. 2). The frequencies of these patterns were 46.67%, 40% and 13.3% for patterns 1 (T/T), 2 (C/C) and 3 (T/C), respectively in normal cyclic animals group whereas these frequencies were 13.33%, 73.33% and 13.33%, respectively in acyclic animals group. The frequencies of alleles T and C were 53.33% and 46.67% and 20% and 80% in normal cyclic and acyclic animals, respectively (Table 2).

Table 2: The pattern frequencies of the *CYP19* gene in thirty tested Egyptian buffaloes

		Pattern Frequencies						Allele frequencies	
		Pattern 1 (TT)		Pattern 2 (C/C)		Pattern 3 (T/C)		Allele T	Allele C
Animals	No. of animals	No.	Frequency	No.	Frequency	No.	Frequency	Frequency	Frequency
Normal cyclic	15	7	46.67%	6	40%	2	13.33%	53.33%	46.67%
Acyclic	15	2	13.33%	11	73.33%	2	13.33%	20.00%	80.00%

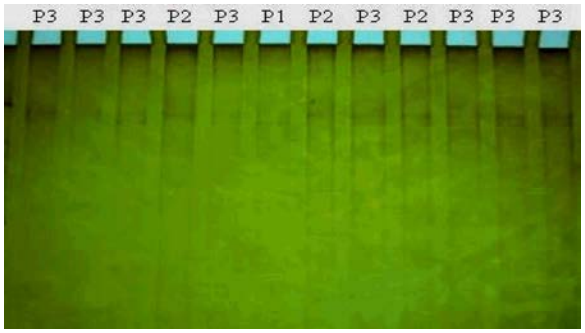

Fig. 2: Three SSCP different patterns of *CYP19* gene in tested Egyptian buffaloes on silver stained-polyacrylamide gel. This figure is not clear, please identify the number of bands in each lanes associated with the three patterns

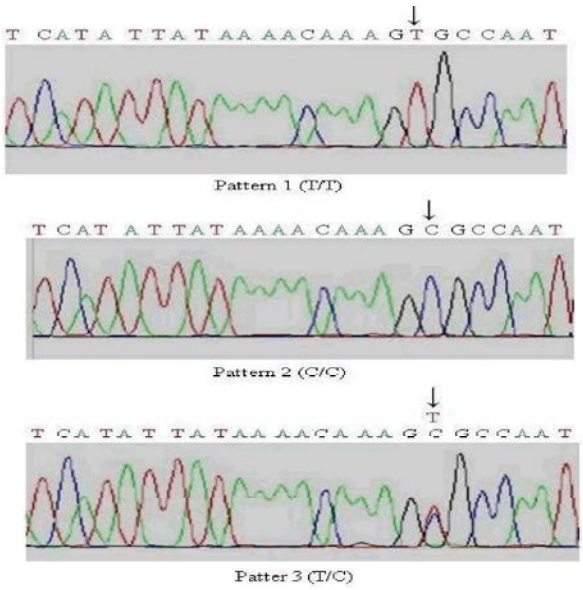

Fig. 3: Single nucleotide polymorphism (T→C) in amplified fragments of Egyptian buffalo *CYP19* gene; pattern 1 (T/T), pattern 2 (C/C) and pattern 3 (T/C)

GGGCTTGCTTGTTTTGACTCGTAACTATAAATTTGTCTTGTATAAGTG
TCCAATCATATTATAAAACAAAGT/CGCCAATCTCTACGGTACAGCA
TCCTCTGAAGCAACAGGAGTCCTGAATGTACATTTTGGGGATTTTCT
AATTTTTCCACTCTTCTGATCTCCACAGGACTTTAAATTACTTCCCCT
GAGATCAAGTAAAACAAAATGCTTTTGGAAGTGCTGAACCCAAGGC
ATTACAATGTCACCAGCATGGTGTCCGAAGTTGTGCCTATTGCTAGC
ATTGCAGTCCTGCTGCTCACTGGATTTCTTCTCTTGGTTTGGAATTAT
GAGGACACATCCTCAATACCAG

Fig. 4: The nucleotide sequence of *CYP19* gene in Egyptian buffalo T/C: Single nucleotide polymorphism between T and C alleles

Sequences of Different Genotypes Patterns of *Cyp19* Gene: PCR products of different genotyping patterns for *CYP19* gene were purified and sequenced to identify the sequences of these different genotypes and to detect SNPs between them (Figs. 3 and 4). The nucleotide sequences of two different alleles C and T of *CYP19* gene in Egyptian buffalo were submitted to GenBank (NCBI, BankIt) and have the accession nos. KJ551928 and KJ551929, respectively.

DISCUSSION

Buffalo is one of the most economically important farm animals in Egypt; it is the major source of milk and meat. One of the limiting factors for quick genetic improvement of Egyptian buffalo is poor reproduction. This study recorded a high prevalence of ovarian inactivity which was confirmed by low undetectable progesterone level. The hormone elevation is considered

as a marker for ovulation and corpus luteum formation. The incidence of ovarian inactivity was as high as 41.53% with higher prevalence in heifers than cows. Affected animals showed poor condition score. Although bad nutrition plays a role for sound ovarian activity [5], the genomic variations have an important role.

Our previous studies on Egyptian cows revealed that the genotypes of blood proteins as Albumin (Al), postalbumin (Pal), α-globulin (Fα), posttransferrin (Ptf), Amylase-1 (Am-1) and transferrine (Tf) were considered as genetic markers of fertility in Friesian cows in Egypt where the high fertility index was related to Al^{A}, Pal^{A}, $F\alpha 2^{A}$,Ptf^{A} and $Am1^{B}$ genes and low fertility index was more related to Al^{B}, Tf^{E} and Pal^{B} genes [13] while in Egyptian buffalo cows, Al^{F}, Pal^{A}, $F\alpha 2^{A}$,$Tf^{D}$and Tf^{E}gene markers were highly correlated with the normal cyclic animals [14].

In the current study, we traced genetic variation and polymorphism with these different phenotypes of ovarian activity. Studying of genetic polymorphisms of genes enables breeders to select the super genetic animals. This could be achieved through genetic improvement programs in livestock by direct selection of genes that affect economic traits through marker-assisted selection (MAS).

CYP19 gene is one of cytochrome P450 genes which are the part of the multigene superfamily, which contains 27 distinct gene families. Aromatase cytochrome P450 enzyme is encoded by the *CYP19* gene [8, 15]. One of aromatase role in mammals is the conversion of androgens to estrogens and is essential for reproduction physiology [16]. Estrogens play a crucial role in physiology of reproduction, cell growth, differentiation, mammary gland development and milk synthesis. Due to the numerous functions that estrogens play in farm animals, their genes are considered as candidate markers for production trait [17].

In this study, PCR-SSCP and nucleotide sequencing were used to identify the different genotypes and SNPs of *CYP19* exon 2 in normal cyclic and acyclic Egyptian buffaloes and to assess the association between these polymorphisms with ovarian activity. SSCP analysis of PCR products is commonly used for detecting single nucleotide polymorphisms and mutations in different species [18-20].

The result declared that the genetic pattern TT is present in normal cyclic animals with higher frequency (46.67%) than that in acyclic animals (13.33%). On the other hand, CC genotype is present with high frequency in acyclic animals (73.33%) whereas its frequency was 40% in normal cyclic animals. Kumar *et al*. [7] reported that TT homozygote is present in control animals, but TT is replaced by TC heterozygote at the same position in late matured and true anestrus animals with completely absence of CC homozygote.

Our sequence finding indicates that the substitution of nucleotide T by C at position 72 in the amplified fragments (covered exon 2) is related to the ovarian activity in Egyptian buffalo whereas these two nucleotides; T and C are present at high frequencies in normal cyclic and acyclic animals, respectively. However, the heterozygote TC is present with the same frequency in the two tested groups (13.33%).

The relation of polymorphic variants of *CYP19* gene with reproductive traits was examined in Polish Holstein-Friesian cows [21]. They reported the high frequency of one homozygous genotype *CYP19/Pvu* II *AA* (0.84780). Longer calving-to-conception intervals and the average calving intervals in *CYP19AA* cows were found, compared to heterozygotes and this difference was significant in the first and third lactations. Kowalewska [22] determined the genetic structure of Polish Holstein-Friesian cow herd based on the polymorphism within *CYP19/Cfr*131 and *CYP19/Pvu*II using PCR/RFLP technique. *CYP19/Cfr*131 allele frequencies were 0.86 for A and 0.14 for B alleles whereas their frequencies in *CYP19/Pvu*II were 0.91 and 0.09 for A and B, respectively.

The association between some important economic traits like milk production and SNPs of the *CYP19*/*Pvu*II in Black-and-White cattle was analyzed [21] where two different alleles were identified; A with high frequency (0.923) and B with low frequency (0.077). This high frequency of allele A confirmed previous results of Jedrzejczak *et al*. [23] who studied this polymorphism *CYP19-Pvu*II in Black-and-White and Jersey cattle. They reported the frequencies of genotypes and alleles for the Black-and-White cows as 0.8985 for AA, 0.0977 for AB, 0.0038 for BB and 0.9474 for A and 0.0526 for B. In the Jersey, all cows were genotyped as *CYP19AA*. They concluded that there weren't any associations between *CYP19-Pvu*II polymorphism and milk production traits in the investigated cows.

CONCLUSSION

The sequences of different alleles for Egyptian buffalo *CYP19* gene indicating that the substitution of nucleotide T by C at position 72 in the amplified fragments (Covered exon 2) may related to the ovarian activity in Egyptian buffalo whereas these two nucleotides T and C are present at high frequencies in normal cyclic and acyclic animals, respectively.

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