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ABSTRACT

The objectives of this study were to detect polymorphisms of LHR and INHIBβA genes and their association with reproductive problems (repeat breeder syndrome) among Egyptian buffalo heifers using PCR–RFLP and DNA sequencing techniques. DNA was collected from 243 (96 normal fertile and 147 repeat breeders) Egyptian buffaloes. PCR-HhaI of 303 bp from LHR gene revealed one uncut TT band confirmed by DNA sequencing showing no SNPs in all the enrolled animals. Meanwhile PCR-StyI of 288 bp from INHIBβA gene yielded two digested (150 and 138 bp) fragments for genotype CC, three fragments (288, 150, 138 bp) for genotype CT and one undigested fragment (288 bp) for TT genotype. Results indicated that out of 96 normal fertile animals, the frequencies of CC, CT and TT genotypes were 32.3%, 39.6% and 28.1% respectively. Meanwhile 15%, 38% and 47% respectively in repeat breeder buffaloes. Frequencies of C and T alleles were 52% and 48% in normal animals and 34% and 66% in repeat breeders. Statistical analysis indicated that there was a significant association between occurance of repeat breeder and INHIBβA genotypes. DNA sequencing declared C/T SNP in intron 1 of INHIBβA gene that significantly appeared with higher frequency in repeat breeder than normal fertile helfers indicating that T allele is a risk factor and can be used in MAS for early selection of repeat breeder heifers, so improving reproductivity of Egyptian buffaloes.

Keywords: LHR, INHIBβA genes, reproductive performance, Egyptian heifers, PCR-RFLP, DNA sequencing.

INTRODUCTION

Egyptian river buffaloes (*Bubalus bubalis*) are cloven hooved mammals, genus *Bubalus* and species *bubalis* **(Perera et al., 2005).** The main four breeds: Menofi, Beheri and Saidi are found in Egypt depending on slight phenotypic differences concerning size, colour and production **(El-Beltagi, 2006)**. Because river buffaloes are the major supply of meat and milk in Egypt, so it serves as an important role in the Egyptian economy **(Kumar et al., 2009).** The farming of buffaloes has long been favored because of their efficient utilization of low quality roughage diet **(Larsson, 2009).** In buffaloes, fertility problems such as repeat breeder is not easily recognized **(Azawi et al., 2008).** This syndrome is responsible for long service period and intercalving interval resulting in greater economic losses in the dairy industry. Anestrus, ovarian inactivity, silent heat and endometritis considered as the main reproductive disorders in Egyptian buffaloes **(Ahmed et al., 2010).** Buffalo heifers that fail

to conceive after 3 or more inseminations with fertile semen and associated with true estrus are classified as repeat breeders **(Purohit, 2008).** Fertility is considered as a very important reproductive trait because it is a compound trait including many important traits **(Moolmuang, 2004)**. A molecular marker is defined as a particular segment of DNA that is representative to the differences at the genomic level, located at specific locations of the genome **(Agarwal et al., 2008). Ota et al., (2007)** mentioned that the importance of PCR-RFLP for rapid finding of point mutations. RFLP markers are greatly polymorphic, their mode of inheritance is codominance and allows for investigating numerous samples **(Linda et al., 2009)**. DNA sequencing acts as a platform for SNP discovery that enabled the detection of mutations and polymorphisms in a genome. Determination of the sequence of a large number of DNA samples rapidly and accurately for recognition of mutations is a critical goal **(Ronaghi and Elahi, 2002).** *LHR* gene is located on bovine chromosome 11 and composes of 11 exons and 10 introns that are positioned in a region coding the extracellular domain of the receptor **(McFarland et al., 1989).** The expression of *LHR* gene in the ovary is induced by *FSH* hormone, estrogen and growth factors in granulosa cells of the preovulatory follicles. It is required for maturation of follicles, ovulation and luteal function in the ovary and it present on granulosa cells, theca cells and luteal cells **(Ascoli et al., 2002)**. Inhibin gene is one of the five main groups within the transforming growth factor beta *(TGF*β*)* superfamily **(Burt and Law, 1994).** It is located at the long arm of the second chromosome in bovines at regions from 36 to 42 **(Barendse et al., 1994**). They are dimeric glycoproteins that are made of a common inhibin alpha subunit that is linked to one of two related subunits: *INHIBβA* and *INHIBβB* **(Ling et al., 1985).** Secreted from granulosa and theca cells of ovary **(Palta et al., 1996). Bernard et al., (2001)** reported that inhibin was named because of its important effect in inhibiting pituitary synthesis and secretion of *FSH*. The physiological role of inhibin in regulation of folliculogenesis in females can be classified into three types: endocrine, paracrine and autocrine **(Knight and Glisten, 2001).** The main objectives of this work were to detect polymorphisms of *LHR* and *INHIBβA* genes and their association with incidence reproductive problems (repeat breeder syndrome) among Egyptian buffalo heifers using PCR–RFLP and DNA sequencing techniques.

MATERIALS AND METHODS

This research was carried out at the Biotechnology Lab, Faculty of Veterinary Medicine, Zagazig University, Egypt.

1–Animals:

This study was conducted on 243 (96 normal fertile and 147 repeat breeder) Egyptian buffaloes (*Bubalus bubalis*). Animals were selected from three localities: A buffalo nucleus herd kept in Nataff-Gedeed Station (20 normal and 72 repeat breeder), Mahalet-Mousa Farm, Agricultural Research Centre, Animal Production Research Institute. Ganat El-Reida (35 and 47 repeat breeder) and El-Noor farms (41 normal and 28 repeat breeder), Ismailia governorate. Blood samples were collected from jugular vein into sterilized vacutainer tubes containing EDTA as an anticoagulant and then stored at −20°C for genomic DNA extraction.

2-Genomic DNA extraction:

Genomic DNA was extracted from the leucocytes using Gene JET Genomic DNA

purification kit following the manufacturer protocol (Thermo Scientific, # K0721/USA). The quality of the extracted DNA was assessed by 1% agarose gel electrophoresis.

3-Polymerase chain reaction (PCR):

Fragments from exon 11 of *LHR* gene (303 bp) and a 288 bp from first intron of the *INHIBA* gene were amplified by PCR using primers shown in **Table 1**. It was carried out in a volume of 50 µl containing 19 µl H_2O, 1.5 µl forward primer, 1.5 µl reverse primer, 3 µl DNA and 25 µl PCR master mix (Bioline, England). The conditions of PCR program was showed in **Table 2**, then PCR products were resolved by electrophoresis, stained with ethidium bromide and visualized using UV light of gel documentation system, model: (UVDI Major Science, USA).

4-Genotyping using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique:

5 µl of the PCR products were digested with restriction enzymes: *HhaI* for *LHR* gene (part of exon 11) and *StyI* for *INHIBA* gene (part of intron 1), incubated at 37°C/5-15 min. The cleaved fragments were detected by 2% agarose gel electrophoresis and visualized under UV using gel documentation system.

5-Statistical analysis:

Gene and genotype frequencies of *LHR* and *INHIBA* genes were calculated according to $p^2+2pq+q^2=1$ **(Falconer and Macky, 1997).** Chi-square (χ^2) used to check whether the population is in Hardy-Weinberg equilibrium or not. The association between infertility and SNPs of the amplified genes was assessed by logistic regression, odds ratio and the corresponding 95% confidence interval.

DNA sequencing:

PCR products with expected size from twenty buffaloes (five normal fertile and five repeat breeders) for each gene were purified using PCR purification kits following the manufacturer protocol (INTRON). The purified PCR products were sent to Sigma company (Germany) for sequencing in forward direction. The obtained sequences were analyzed using Chromas software. Sequence analysis and alignment were carried out using NCBI/BLAST and CLC Main Workbench7 software**.**

Table 1: Forward and reverse primers sequence, annealing temperatures and size of PCR amplicon (bp) for *LHR* and *INHIBA* genes:

Gene	Primers		Annealing temperature (°C)	Size of PCR product (bp)	Reference
	Forward (5′-3′)	Reverse (5′-3′)			
LHR (part of exon 11)	5′-CAAACTGACAG TCCCCCG CTTT-3′	5′-CCTCCGAG CATG ACTGG AATG GC-3′	57	303	**Marson et al., (2008)**
INHIBA (part of intron 1)	5′-GGTGGTTGTTA CTGTTTATC-3′	5′-CAGGGTTTCAG AAGTTGG-3′	55	288	**Sang et al., (2011)**

Table 2: Conditions of polymerase chain reaction (PCR) for *LHR* and *INHIBA* genes:

Gene	Initial denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extension
LHR (part of exon 11)	94/5 min	35	94/30 s	57/30 s	72/30 s	72/8 min
INHIBA (part of intron 1)	95/5 min	40	95/30 s	55/30 s		

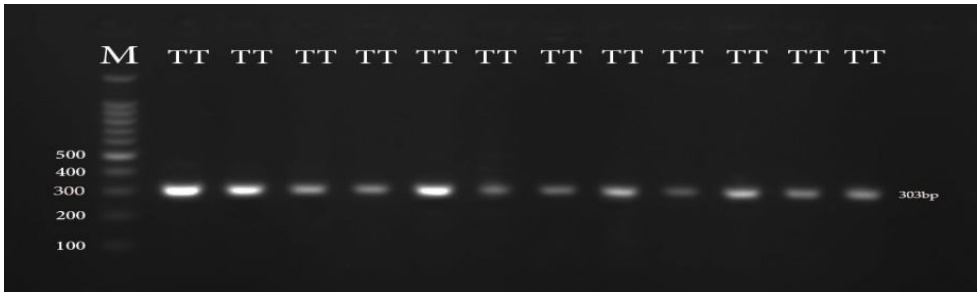

Figure 1: Ethidium bromide stained 2% agarose gel electrophoresis of representative samples of RFLP banding pattern of *LHR* gene (part of exon 11-303 bp) from normal fertile and repeat breeder buffaloes after digestion with *HhaI*. M: 100 bp ladder.

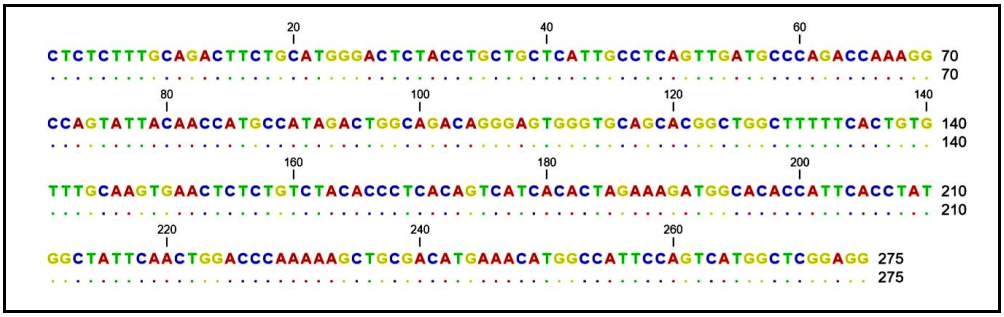

Figure 2: DNA sequence alignment of *LHR* gene (part of exon 11-303 bp) between normal fertile and repeat breeder buffaloes using CLC Main Workbench7 program showing no variation between two groups.

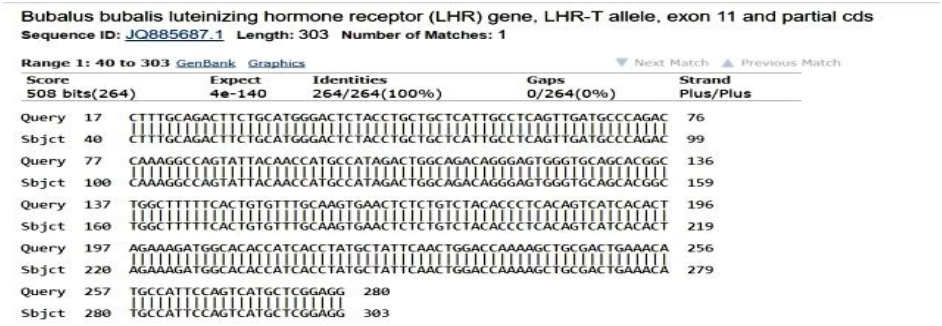

Figure 3: Nucleotide sequence alignment of *LHR* gene from normal fertile buffaloes using BLAST showing 100% identity with *Bubalus bubalis* and no variation between the studied animals.

Figure 4: Ethidium bromide stained 2% agarose gel electrophoresis of representative samples of RFLP banding pattern of *INHIB-βA* gene from normal fertile and repeat breeder buffaloes after digestion with *StyI*. M: 100 bp ladder.

Table 3: Genotypic and allelic frequencies of the *INHIBβA* gene (288 bp) in normal fertile and infertile animals.

Animals	No. of animals	Number/frequency of genotypes %			Allele frequency %		Risk allele	χ2 (HWE)	P-value
		CC	CT	TT	C	T			
Fertile	96	31/32.3	38/39.6	27/28.1	52	48	T	4.112	0.04259
Infertile	147	22/15	56/38.1	69/46.9	34	66		3.367	0.06652

HWE – Hardy Weinberg Equilibrium. Hardy Weinberg test was done using the Pearson's goodness of fit test. P value<0.05 was considered to show significant deviation of the observed genotypes from Hardy-Weinberg proportions.

Table 4: Genotypic and allelic association of C>T SNP of INHIBβA gene (288 bp) polymorphism with infertility under different genetic models.

Comparative models	**Genotypes**	**Fertile (n=96)**	**Infertile (n=147)**	**OR (95% CI)**	**P-value**
Codominant	T/T	27 (28.1%)	69 (47 %)	1.00 (reference)	0.016
	C/T	38 (39.6 %)	56 (38 %)	0.58 (0.31-1.06)	
	C/C	31 (32.3 %)	22 (15 %)	0.28 (0.14-0.56)	
Dominant	T/T	27 (28.1 %)	69 (46.9 %)	1.00 (reference)	0.003
	C/T-C/C	69 (71.9 %)	78 (53.1 %)	0.44 (0.26-0.77)	
Recessive	T/T-C/T	65 (67.7 %)	125 (85 %)	1.00 (reference)	0.0015
	C/C	31 (32.3 %)	22 (15 %)	0.37 (0.20-0.69)	
Overdominant	T/T-C/C	58 (60.4 %)	91 (61.9 %)	1.00 (reference)	0.82
	C/T	38 (39.6 %)	56 (38.1 %)	0.94 (0.55-1.59)	
Log-additive	---	---	---	0.53 (0.37-0.75)	0.0003
INHIB-βA C>T	Allele	Fertile (n=96)		Infertile (n=147)		OR (95 % CI)	P-value
		No.	%	No.	%		
	C	100	52 %	100	34 %	1.00 (reference)	< 0.0001
	T	92	48 %	194	66 %	2.11 (1.45-3.06)	

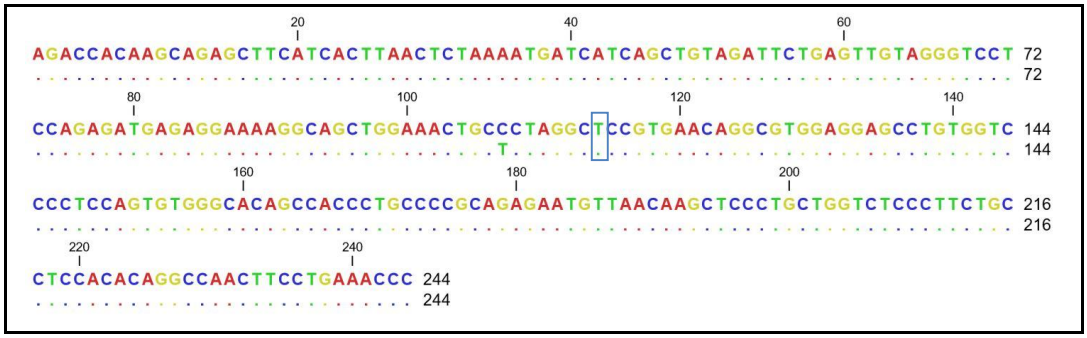

Figure 5: DNA sequence alignment of *INHIBβA* gene (part of intron 1-288 bp) between normal fertile and repeat breeder buffaloes using CLC Main Workbench7 program showing C/T transition.

Score	Expect	Identities	Gaps	Strand
254 bits(137)	7e-72	140/141(99%)	1/141(0%)	Plus/Plus

```
Query  115  CTAGGCTCCGTGAACAGGCGTGGAGGAGCCTGTGGTCCCCTCCAGTGTGGGCACAGCCAC  174
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    CTAGGCTCCGTGAACAGGCGTGGAGGAGCCTGTGGTCCCCTCCAGTGTGGGCACAGCCAC  60

Query  175  CCTGCCCCGCAGAGAATGTTAACAAGCTCCCTGCTGGTCTCCCTTCTGCCTCCACACAGG  234
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CCTGCCCCGCAGAGAATGTTAACAAGCTCCCTGCTGGTCTCCCTTCTGCCTCCACACAGG  120

Query  235  CCAACTTCCTGAAACCCTGAA  255
            ||||||| ||||||||||||
Sbjct  121  CCAACTT-CTGAAACCCTGAA  140
```

Figure 6: Nucleotide sequence alignment of *INHIBβA* gene (part of intron 1) from normal fertile buffaloes and *Bos taurus* sequence with accession number rs43408735 **(Sang et al., 2011)** using BLAST showing 99% identity.

RESULTS AND DISCUSSION

LHR gene:

PCR-*HhaI* of *LHR* gene revealed mononmorphic pattern (uncut band) at 303 bp in all the studied animals as revealed in **Figure 1.**

Alignment of 303 bp of *LHR* gene from normal fertile and repeat breeder buffaloes were performed using CLC Main Workbench7 program showing no variation between two groups and presented in **Figure 2**. The results of nucleotide sequences confirmed the uncut pattern of PCR-RFLP and no variation exists between the sequence of the studied animals.

While Alignment from normal fertile buffaloes with *Bubalus bubalis* using BLAST with JQ885687.1 accession number shows 100% identity between them and is revealed in **Figure 3.**

LH interacts with *LHR* to affect various activities in the body such as follicular growth, oocyte maturation, ovulation and corpus luteum formation which are necessary for reproductive function of the females **(Hyttel et al., 1997).** The results of this context agree with **Othman and Abdel-Samad, (2013)** who digested 303 bp of *LHR* gene yielding uncut pattern in all animals and **Sosa et al., (2016)** that found no polymorphism between fertile and non fertile buffaloes and they possessed 100% identities with Egyptian buffaloes. On contrary, these results disagree with those obtained by **Marson et al., (2008)** who worked to associate between RFLP-*HhaI* of *LHR* gene and its influence on propability of pregnancy in cattle heifers. They found three genotypes: TT, CT and CC. The heterozygous heifers CT showed a higher pregnancy rate than TT and

CC homozygous ones. However, the effect of the *LHR* gene polymorphism on pregnancy was not accured. **Yang et al., (2012)** used PCR-SSCP and obtained GG, GT and TT genotypes and found that heifers with GG and GT genotypes had a significant increase in the total number of ova than TT genotype. **Kumar et al., (2014)** disagree with our results, where SNP variation at 243 bp nucleotide position of *LHR* gene was reported which did not show any significant correlaration with postpartum anestrus. The results of **Arslan et al., (2017)** are not in the same line with the obtained results. Where they found three genotypes of *LHR* gene after digesting with *HhaI* enzyme producing a significant deviation from HWE.

***INHIBβA* gene:**

PCR-*StyI* of *INHIBβA* gene revealed three genotypes: TT genotype with one undigested fragment at 288 bp, three digested fragments at 288, 150 and 138 bp for genotype CT and two digested fragments at 150 and 138 bp for genotype CC from normal fertile and repeat breeder buffaloes as revealed in **Figure 4.**

The genotypic and allelic frequencies of *INHIBβA* gene were calculated and presented in **Table 3**. For 96 normal fertile buffalo cows genotypic frequencies of CC, CT and TT genotypes were 32.3%, 39.6% and 28.1% respectively, while allelic frequencies of the C and T alleles were 52% and 48%. In 147 infertile repeat breeder heifers the genotypic frequencies of CC, CT and TT genotypes were 15%, 38 % and 47% respectively and allelic frequencies of the C and T alleles were 34% and 66%. The χ2-test presented the obtained *INHIBβA* genotypic distribution among normal fertile buffaloes that was deviated from Hardy Weinberg equilibrium ($p<0.05$), while in repeat breeder heifers the genotypic distribution follow Hardy Weinberg equilibrium ($p>0.05$).

As presented in **Table 4**, logistic regression analysis revealed that a significant association of C and T alleles of *INHIBβA* gene with repeat breeder ($P<0.0001$). In repeat breeder buffaloes, T allele showed a higher infertility percent (66%) compared to C allele (34%). This result means that buffaloes carrying the risk allele T which has a higher susceptibility to infertility in comparison to C allele carriers and increased the OR value =2.11 with 95% CI= 1.45-3.06 of the risk for infertility. While in normal fertile heifers T allele appeared with lower frequency (48%) comparing to C allele (52%). The occurance of C/T transition SNP showed a significant association when tested using various genetic models. However, with the codominant model ($P=0.016$), animals with a homozygous genotype TT at this locus had an OR of 1.00 with 95% CI for being repeat breeder compared to CT genotype that had OR = 0.58 with 95% CI= 0.31-1.06, while CC genotype had OR of 0.28 and 95% CI= 0.14-0.56. Under the dominant model of C>T SNP (T/T versus C/T+C/C) showed a highly significant association ($P=0.003$) with repeat breeder with OR of 0.44 and 95% CI= 0.26-0.77. The recessive model of SNP (T/T+C/T versus C/C) showed significant association ($P=0.0015$) with repeat breeder with OR of 0.37, 95% CI= 0.20-0.69. The overdominant model (T/T+C/C versus C/T) revealed that a non significant association ($P=0.82$) with repeat breeder with OR of 0.94 and 95% CI= 0.55-1.59. The log-additive effect ($P=0.0003$) can be interpreted as every additional copy of the minor allele T at this locus resulted in an increased risk of repeat breeder by 0.53, 95% CI= 0.37-0.75 in buffaloes.

Alignment of 288 bp of *INHIBβA* gene in normal fertile and repeat breeder buffaloes

were performed using CLC Main Workbench7 program showing C/T SNP at base 107 of intron 1 that increase susceptibility of heifers to repeat breeder syndrome as shown in **Figure 5.**

While nucleotide sequences alignment of *INHIBβA* gene in normal fertile buffaloes with *Bos taurus* sequence with accessation number rs43408735 of **Sang et al., (2011)** was performed using BLAST and presented in **Figure 6** that showed 99% identity.

It is noticeable that C/T transition SNP had a significant effect on reproductivity of buffalo heifers, as it increases the susceptibility of occurance of repeat breeder. The present results are in line with those reported by **Ke-Qiong et al., (2011)** who reported the association between *INHIBα* gene polymorphism and superovulation traits in cattle. Where the allelic frequencies of A and G alleles are 0.551 and 0.449 between animals. Also, **Sang et al., (2011)** digested 288 bp of *INHIBβA* gene with *StyI* endonuclease resulting in three genotypes CC, CT and TT. C/T mutation of *INHIBβA* gene had a significant effect on volume and sperm concentration in cattle bull. Although the C/T SNP is located in the intronic region of *INHIBβA* gene that does not translated into protein and so does not changed the amino acid, but it is near to the exon-intron junction where it was important for mRNA splicing **(Pang et al., 2011).** On another hand, **Tang et al., (2011)** detected A192G mutation in exon 2 of *INHIBα* gene in Chinese Holstein cattle. Although it was a synonymous mutation that did not change the amino acid as both CGA and CGG codons encode arginine amino acid but it affected the expression of the *INHIB* gene and the stability of the transcript so increased the circulating concentration of *FSH* and improved the reproductive performance. **Chu et al., (2012)** amplified fragment of *INHIBβA* gene using two primers and obtained three genotypes AA, AB and BB in Jining Grey and two AB and BB in Inner Mongolia Cashmere and Angora goats. Sequencing revealed one A/G single nucleotide mutation at base 782 of exon 2 of *INHIBβA* gene in BB genotype compared to genotype AA. On contrary to the obtained results, **Yang et al., (2014)** used *StyI* endonuclease to digest 288 bp of *INHIBβA* and resulted in three genotypes CC, CT and TT. The authors reported that the C/T substiuation did not affect superovulation traits in Chinese Holstein cows and the genetic polymorphism was followed in Hardy Weinberg equilibrium.

CONCLUSION

This study highlights the significant association between *INHIBβA*/*StyI* locus polymorphism and fertility traits in Egyptian buffaloes by using PCR-RFLP and DNA sequencing genetic markers more than *LHR*/*HhaI*. Moreover, *INHIBβA*/*StyI* gene-T allele can be used as a marker for early selection of repeat breeder animals and early culling of low fertile heifers with TT higher risk geneotype resulting in preventing economic losses afforded by the latter and improve productivity of buffaloes in Egyptian farms.

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الملخص العربي
الإرتباط بين الإختلافات فى جيني مستقبل هرمون التبويض ومانع التبويض وصفات الخصوبة في إناث الجاموس المصري

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أجريت هذه الدراسة من أجل معرفة العلاقة بين الإختلاف الوراثي في جيني هرمون التبويض ومانع التبويض والمشاكل التناسلية (تكرار الشياع) بين عجلات الجاموس المصري باستخدام تقنية إختلاف أطوال تقطيع الشريط النووي ومعرفة التتابع النيوكلوتيدي لشريط الحامض النووي. تم عزل الحامض النووي من ٢٤٣ (كان منهم ٩٦ طبيعي و ١٤٧ يعانون من حالات الشياع المتكرر) من عجلات الجاموس. في هذة الدراسة تم عمل تقنية إختلاف أطوال تقطيع الشريط النووي عن طريق استخدام إنزيم القطع HhaI من أجل هضم ٣٠٣ قاعدة نتيروجينية لجين هرمون التبويض (٣٠٣ قاعدة نيتروجينية) والذي لم يقطع هذة القطعة في جميع الحيوانات تحت الدراسة وكانت ذات التركيب الوراثي TT وهذة النتيجة تم تأكيدها عن طريق عمل تتابع نيوكلوتيدي لشريط الحامض النووي من الحيوانات الطبيعية وذات الشياع المتكرر وقد أكد عدم وجود أي اختلافات بين هذه الحيوانات. بينما بالنسبة لجين مانع التبويض فقد تم استخدام تقنية إختلاف أطوال تقطيع الشريط النووي عن طريق استخدام إنزيم القطع StyI من أجل هضم ٢٨٨ قاعدة نيتروجينية وقد وجد أنه قطع الحزمة ٢٨٨ إلى حزمتين (١٥٠ و١٣٨قاعدة نيتروجينية) وهذة العجلات أخدت التركيب الوراثي CC ،ثلاثة حزم (١٣٨ ،١٥٠و٢٨٨ قاعدة نيتروجينية) وهذة العجلات أخدت التركيب الوراثي CT وعجلات أخرى لم يتم قطع الحزمة ٢٨٨ قاعدة نيتروجينية وقد أخدت التركيب الوراثي TT . وقد أظهرت النتائج أن معدل التراكيب الوراثية CC ،CT، TT في ٩٦ عجلة طبيعية كانت ٣٢,٣٪ ،٣٩,٦٪ و ٢٨,١٪ بينما كانت ١٥٪ ،٣٨٪ و٤٧٪ في ١٤٧عجلة ذات شياع متكرر. كان معدل الاليلات C وT هو ٥٢٪ و ٤٨٪ في العجلات الطبيعية بينما ٣٤٪ و ٦٦٪ في العجلات ذات الشياع المتكرر. ومن خلال التحليل الإحصائي وجد أن هناك ارتباط معنوي بين حدوث ظاهرة تكرارالشياع والتراكيب المختلفة لجين مانع التبويض. ومن خلال عمل تتابع نيوكلوتيدي لشريط الحامض النووي تبين وجود طفرة قد غيرت القاعدة النيتروجينية السيتوزين إلى

ثايمين في البادئ الأول من جين مانع التبويض وقد ظهرت بمعدل كبير في العجلات متكررة الشياع مما يبرهن على أن أليل الثايمين هو مؤشر خطر يزيد من إحتمالية التعرض للشياع المتكرر ومن الممكن إستخدامه للإنتخاب المبكر للحيوانات ذات الشياع المتكرر واستبعادها ومن ثم تحسين العملية الإنتاجية في الجاموس المصري.