*Egypt. J. Vet. Sci.***Vol. 48.** No, 1 pp 1- 9 (2017)

Effect of Body Condition Score and PCR-RFLP Polymorphism of Prolactin Gene on Semen Characteristics of Buffalo Bulls (BubalusBubalis)

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THIS WORK aimed to evaluate effect of body condition score on semen characteristics of buffalo bulls and identify the genetic polymorphisms of prolactin (PRL) gene as bases for selection of bulls with good breeding value.The study was performed on 60 buffalo bulls aged 2-8 years. The bulls were classified according to body condition score (BCS) into two groups. The first group of bulls was between 2 to <3 scores (n 33), while the second group was between 3 to <4 scores (n 27).Three semen collections were obtained from each animal at 15-day intervals and evaluated for volume, individual motility, live sperm % and chromatin integrity. DNA was extracted from semen for genotyping of PRL gene XbaI locus of regulatory region using PCR-RFLP technique. The results showed that, body condition score had no significant effect on semen characteristics. XbaI PCR-RFLP of PRL gene showed genetic polymorphism in 678 bp PCR fragment including two genotypes AA and BB.The genotype AA (678 bp) had high frequencies (91.67%) while the genotype BB (231 and 447 bp) had low frequencies (8.33%). There were no association between the two genotypes of prolactin gene and the studied semen parameters. It could be concluded that, BCS of buffalo bulls had no effect on semen quality. The presence of genetic polymorphism in prolactin XbaI locus of buffalo bulls may acts as a genetic marker using large number of animals.

Key words: Buffalo, Semen, PRL gene, PCR, RFLP.

Introduction

Semen evaluation is of high diagnostic importance for assessing testicular and epididymal function, and/or the male genital tract to eliminate the prominent cases of infertility, or potential sub-fertility [1,2],thereby the main goal of semen evaluation is to predict its fertilizing and cryopreservation ability for artificial insemination to obtain acceptable pregnancy rates [3,4]. Recently AI became a tool of disseminating superior genes for economic traits like milk production [5-7]. Sperm DNA integrity may be a good marker for measurement of male fertility potential [8,9]. Rasul et al. [10] reported that, freezing and thawing of buffalo semen may cause a considerable damage to DNA, acrosomal cap, plasma membrane, and motility apparatus, hence evaluation of DNA integrity is important in the design of new freezing-thawing steps

There are several hormones that regulate lactation and reproduction in bovines. One of them is prolactin, which is a polypeptide hormone synthesized and secreted from primarily the lactotrophes of the anterior pituitary gland in bovines and other vertebrates [11]. PRL gene is expressed in the pituitary gland, and at several other sites including the mammary gland, the central nervous system and the immune system [12,13], that allows PRL to have multiple biological functions related to reproduction, osmoregulation, integument growth and synergism with steroids [14]. Additionally, Lazebnaya et al. [15] concluded that, the binding of the bPRL gene product,after its expression, with its receptor initiates a signaling cascade that activates the transcription of a number of genes, including the genes of milk proteins (caseins and lactalbumin).

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DOI: 10.21608/ejvs.2017.868.1011

It was reported that, bovine prolactin gene (bPRL) is located on chromosome 23 in the bovine genome [14,16]. The bPRL gene size is about 10 kb and consists of five exons and four introns [17], that encodes 199 amino acids mature protein [18].The bPRL gene is considered as a common candidate gene for marker-assisted selection for milk production parameters [19].The polymorphisms of prolactin gene and its relationship with milk related traits were confirmed in various cattle breeds [20-26]. However, few studies were done on genotyping this hormone in buffalo [27-29]. It was shown that polymorphism in the structural or regulatory sequences of the PRL gene would be significant to study due to its possible direct and indirect role on milk production traits [29]. So this study aimed to evaluate effect of body condition score on semen characteristics and to identify the genetic polymorphisms of prolactin gene in buffalo bulls.

Materials and Methods

Animals

The study was performed on 60 buffalo bulls (Bubalus bubalis) aged 2-8 years. Buffalo bulls were subjected to semen evaluation and genetic characterization of PRL gene. These bulls belonged to a breeding station at Mahalet Mussa near to Sakha, Kafr el-Sheikh Governorate. All buffalo bulls were subjected to the same management and nutrition programs. The buffalo bulls were classified according to body condition score (BCS) into two groups using a grading scale of 1-5, according to a system for buffalo stated by Alapati et al. [30] where score 1 indicated severe under-condition whereas score 5 indicated severe over-condition (obesity). The first group of bulls was between 2 to <3 scores (n=33) while the second group were between 3 to <4 scores (n=27).

Semen evaluation

Volume of the ejaculate was estimated to nearest 0.1 ml. Individual motility was expressed as the percentage of forward motile spermatozoa. Live sperm percentage was estimated using eosin-nigrosin stained smears. Chromatin integrity was recorded using acridine orange (AO) staining technique [9, 31].

DNA extraction from sperm cells

DNA was extracted from fresh semen according to Weyrich [32] with slight modifications. Fifty µl of semen was washed in 500 µl of 70 % ethanol then the samples were centrifuged for 5 min at 10,000 ×g and the supernatant was removed. The former steps were repeated until the supernatant became clear and an easily visible and a white pellet was obtained. About 500 µl lysis buffer (50 mMTris-HCl , pH 8; 10 mM EDTA; 100 mMNaCl; 1 % SDS aqueous solution) was added to the sperm pellet. Also, 5 µl Triton-X100 (0.5 %), 25µl Dithiothreitol (DTT) 1M and 50 µl proteinase K (20mg/ml) were added. The samples were mixed well and incubated at 50 °C overnight in a thermo shaker. The tubes were centrifuged for 10 min at 15,500 × g and the supernatant was transferred into a new 1.5 ml tube. Sodium acetate (NaAc) 3M was added to the supernatant (bout 1/10 Vol. of the supernatant) and mixed gently. NaAc binds to the DNA and eases the precipitation. An ice cold absolute ethanol was added to the tubes (2 Vol. of the supernatant). DNA was precipitated at -20°C overnight and pelleted by centrifugation for 20 min. at 15,500 x g. The supernatant was carefully removed by pipetting and the white pellet of DNA remained. DNA was washed by dispensing the pellet in 500 µl ethanol (75 %). The samples were centrifuged for 10 min at 15,500x g at room temperature (25-30 °C). The samples were dried until ethanol was evaporated. Avoid drying the pellet completely because it may affect DNA. DNA pellets were dissolved in 50 µl ddH_2O and concentration was measured using NanoDrop1000 Thermo Scientific spectrophotometer then diluted to working concentration of 50 ng/µl, which is suitable for PCR.

Polymerase Chain Reaction (PCR) and DNA amplification

The DNA fragment of PRL gene was amplified through polymerase chain reaction technique developed by Mullis et al.[33].The PCR mixture consisted of 12.5 µl of PCR master mix (2X) composed of 0.1 U/ µl Taq polymerase, 500 µM of dNTP each, 20 µM of Tris-Hcl (pH 8.3), 100 mM of Kcl, 3 mM of MgCl, stabilizer and enhancer. In addition to 1.0 µl of forward primer F(20pM/ µl), 1.0 µl of reverse primer R(20pM/ µl), 2.0 µl of DNA(50 ng /µl) and add water up to 25 µl. The sequence of the primers used were F:5′ AGGTTAGGAGGATAG-3′ and R: 5'TTAGTCAAGTTAGATACCG-3′ according to Li et al. [26] for amplification of 678 bp fragment in 5'regulation region of PRL gene. PRL gene PCR program for amplification was initial denaturation 95 °C for 3 min., then 32 cycle of denaturation 95 °C for 1 min., then an annealing

at 50.6 °C for 1 min., then an extension at 72°C for 1 min. and a final extension at 72°C for 5 min. The PCR reaction products were shown on 2% agarose gel via electrophoresis and stained with red safe to be visualized on UV transilluminator.

Restriction fragment length polymorphism (RFLP)

The PCR products for PRL gene were digested with XbaI restriction enzyme. The restriction mixture for each sample was prepared by adding 2.0 µl of 10 × restriction buffer to 1.0 µl of XbaI restriction enzyme and the volume was completed to 20 µl by dd H_2O then mixed with 10 µl PCR product. Fast digest restriction enzyme was used. This restriction mixture was incubated at 37°C for 25 min. The digested PCR products were showed on 2% agarose gel by electrophoresis and stained with red safe to be visualized on UV transilluminator.

Statistical analysis

The obtained data were expressed as mean ± SE.The effect of body condition score and genotypes of prolactin gene on the studied semen parameters were analyzed by *t*-test using SPSS version 16 software. The differences were considered to be significant at $P < 0.05$.

Results

Semen evaluation

Data in Table 1 showed a non-significant effect of body condition score on fresh semen characteristics of buffalo bulls except for live sperm percentage. The averages of ejaculate volume (ml), individual motility percentage, live sperm percentage and sperm cells with chromatin damages were 2.76±0.17, 78.26±0.85, 82.16±0.68 and 0.70±0.13 respectively in bulls with body condition score between 2 to <3. Meanwhile, the corresponding values for the bulls with body condition score between 3 to <4 were 2.61±0.07, 78.10±0.87, 80.30±0.60 and 0.86±0.14 respectively.

TABLE 1. Effect of body condition score on fresh semen characteristics of buffalo bulls (Mean ±SE).

Body condition score	Ejaculate volume (ml)	Individual Motility %	Live sperm %	Chromatin damage %
2 to <3 (33 animal)	2.76±0.17	78.26±0.85	82.16±0.68*	0.70±0.13
3 to <4 (27 animal)	2.61±0.07	78.10±0.87	80.30±0.60	0.86±0.14

*p<0.05 (t-Test).

Genotyping of Prolactin (PRL) gene

A total number of 60 buffalo bulls were tested for polymorphism in PRL gene using PCR–RFLP technique. All the tested buffalo bulls gave PCR specific band at the expected size 678 bp from 5 regulatory region of PRL gene (Fig. 3).

By using RFLP technique, these PCR amplified fragments of 678-bp were digested with *XbaI* restriction endonuclease depending on the presence or absence of the restriction site at position 231^447 and gave two patterns of genotypes AA and BB. Pattern AA is the amplified PCR product of 678 bp from buffalo remained undigested by *XbaI* restriction enzyme while pattern BB when the amplified PCR product of 678 bp digested by *XbaI* restriction enzyme into two fragments 231 and 447 bp (Fig. 4).

Results displayed the presence of AA genotype in high frequencies (91.67%) where 55 animals from 60 were AA while BB genotype recorded low frequencies (8.33%) where only 5 animals from 60 were genotyped as BB (Table 2). Hence, the allele frequencies also indicated more prominent allele A and less prominent allele B in the studied bulls. These results showed a polymorphic pattern of that fragment of this gene with presence of genetic variation in Egyptian buffalo bulls studied population.

Regarding the effect of genotypes of prolactin gene (AA and BB) on characteristics of buffalo semen, the ejaculate volume (ml), individual motility %, live sperm % and chromatin damage % were 2.45±0.11 and 2.90±0.35 ml, 75.40±1.48 and 76.80±2.14, 81.50±1.55 and 79.80±1.42 and 0.85± 0.37 and 1.30±0.47 for the genotype AA and genotype BB respectively (Table, 3).Statistical analysis revealed no significant differences in all parameters studied between the two genotypes of prolactin gene.

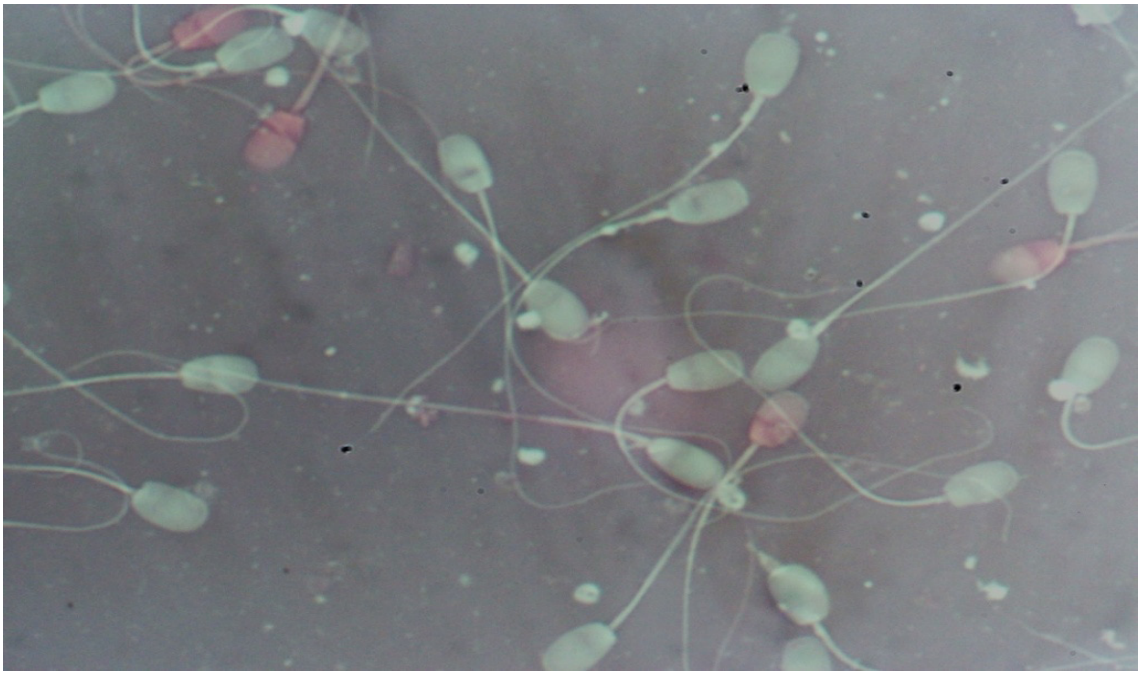

Fig.1. Light microscopy for buffalo spermatozoa stained by eosin-nigrosin stain, live sperm appeared white and dead sperm appeared pink or red color of eosin (x1000).

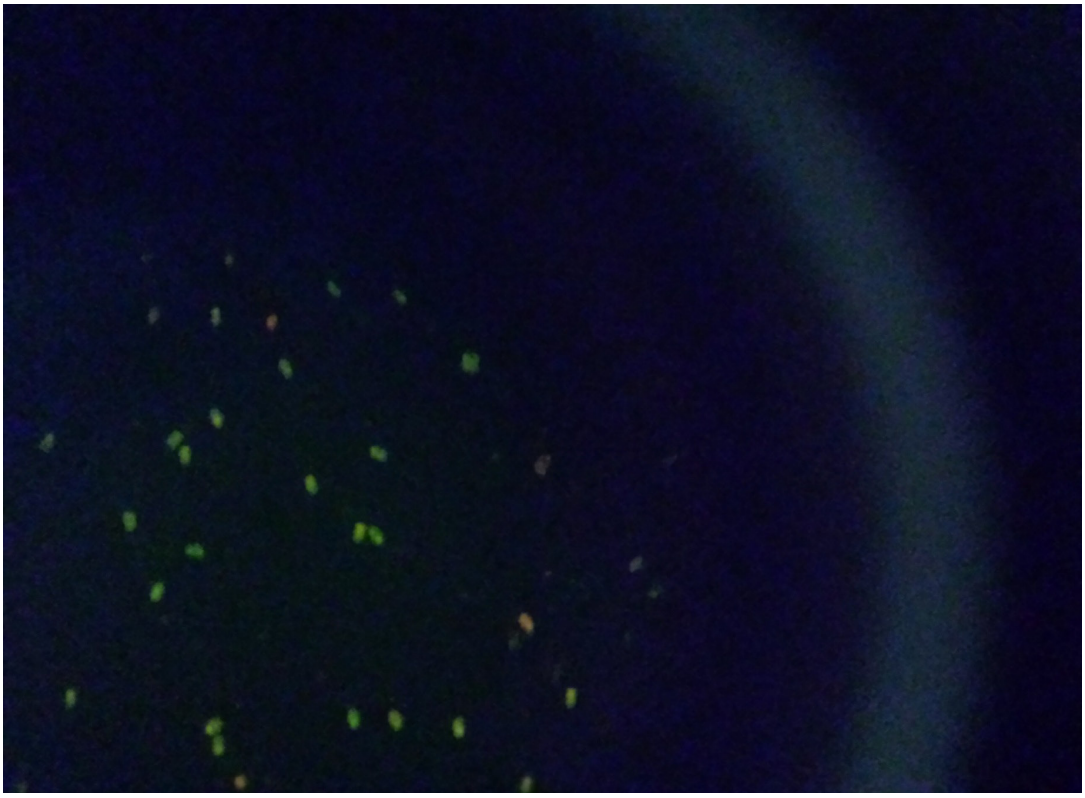

Fig.2 . Fluorescence microscopy for buffalo spermatozoa stained by acridine orange stain, damaged chromatin appeared yellow or red color of acridine orange stain and normal chromatin appeared green color (×400).

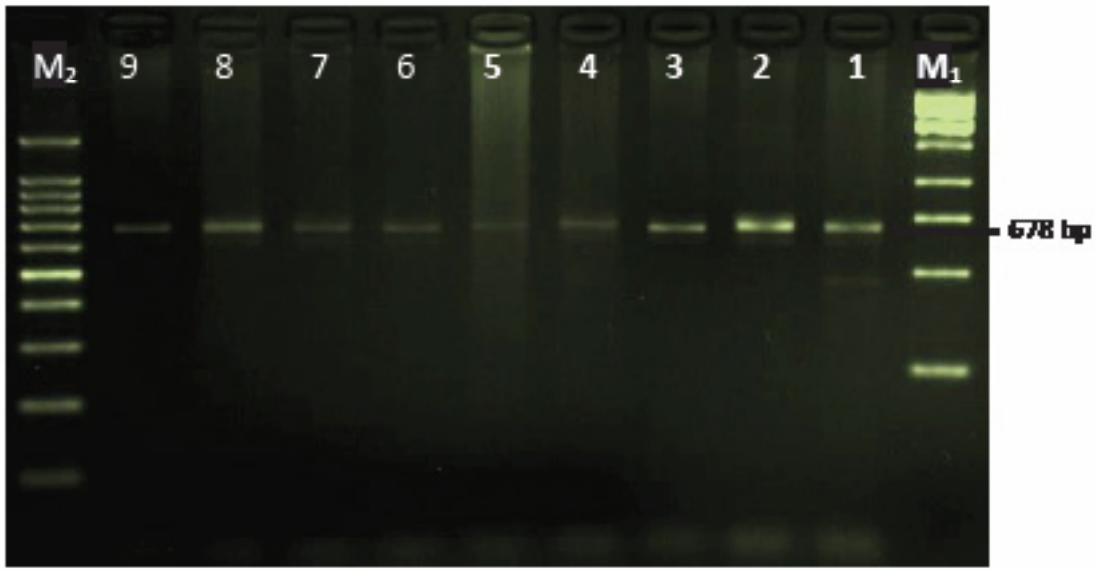

Fig. 3. Red safe-stained gel of PCR products representing amplification of PRL gene in buffalo bulls visualized on 2% agarose gel. M_1: 250-bp ladder marker. Lanes 19- resemble 678-bp PCR products. M_2: 100-bp ladder marker.

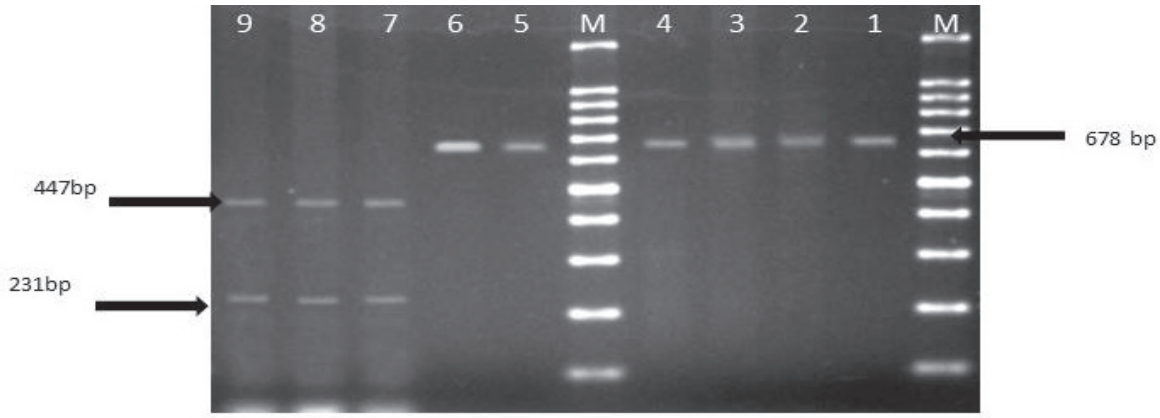

Fig. 4. Red safe-stained 2% agarose gel of RFLP products for PRL PCR digested with *XbaI* enzyme. M: 100-bp ladder. Lanes 1-6 showed AA genotype of 678 bp, lane 7-9 showed BB genotype of two digested fragments at 231 and 447 bp.

TABLE 2. Frequencies of different genotypes of prolactin gene in buffalo bulls.

Genotype	Number of bulls	Genotype frequency
AA	55	91.67 %
BB	5	8.33 %

TABLE 3. Effect of genotypes of prolactin gene on characteristics of buffalo semen (Mean ±S.E).

Semen characteristics	Genotype	
	AA	BB
Ejaculate volume (ml)	2.45±0.11	2.90±0.35
Individual motility %	75.40±1.48	76.80±2.14
Live sperm %	81.50±1.55	79.80±1.42
Chromatin damage %	0.85± 0.37	1.30±0.47

Discussion

According to our results, the body condition score has no significant effect on fresh semen characteristics of buffalo bulls. Similarly, Sarder [34] pointed that, there were no significant effects of body condition score of bulls on some semen characteristics. Meanwhile, Addass [35] reported that, there were significant effects of body condition score of bulls on sperm production potential and scrotal circumference. Also Beran et al. [36] observed significant effects of body condition score of bull on the sperm motility, sperm concentration and percentage of abnormal sperm.

Okere et al. [37] studied the relationships among body condition score, body weight, testicular traits and semen output in electro-ejaculate pubertal kiko goat bucks.There were low and negative correlations between body condition score and body weight and semen volume indicating that semen output is independent of body condition score and body weight. Moreover, Akpa et al. [38] observed that, body condition score of red Sokoto goat had no significant influence on sperm motility, live and dead ratio and sperm concentration but it had a significant influence on semen volume and semen PH.

In mammals especially dairy cattle, the prolactin gene has important functions like the development of mammary gland and affecting milk yield and composition [39]. *PRL* gene seems to be promising potential candidate gene associated with dairy performance traits and used in marker-assisted selection of dairy farm animals because it plays a crucial role in mammary gland development and in the initiation and maintenance of lactation and expression of milk protein genes [15].In the present study, genetic polymorphism was recorded in PCR product of 678 bp fragment from 5 regulatory region of prolactin gene in the studied buffalo bulls with presence of AA genotype in high frequencies while BB genotype in low frequencies, which indicates a presence of genetic variation in Egyptian buffalo bulls in that fragment of this gene.Nearly the same results were recorded previously by Li et al. [26] who concluded that, the BB genotyped cows yielded significantly higher milk yield, milk protein and milk fat than AB cows. Meanwhile, no significant effects of the AA genotype on the above traits.

Kumari et al. [39] mentioned that a silent A→G transition mutation in exon 3 of bovine prolactin gene that encodes amino acid 103 which created a polymorphic *Rsa*I site. Also, Dybus [40] studied the associations of prolactin genes polymorphic *Rsa*I site with milk production traits in Polish Black and White cattle. He recorded genotype frequencies of 0.734 for AA, 0.257 for AB and 0.009 for BB. He noticed that AA cows produce milk with higher protein content than AB and BB individuals. In addition, Boleckova, *et al.*[41] recorded the association of PRL *Rsa*I polymorphisms with milk production traits in Czech Fleckvieh cattle. The genotype frequencies were 0.01 for AA, 0.22 for AG and 0.77 for GG fragment with allele frequencies of 0.12 for A and 0.88 for G allele. Besides that, Mehmannavaz *et al.*[22] analyzed the *PRLRsa*I polymorphisms in Iranian Holstein bulls and the frequencies recorded for A and G alleles were 0.069 and 0.931, respectively. The allelic substitution effect for A was significant for milk and protein yield ($p < 0.05$) where the G allele was unfavorable for milk and protein yield. Moreover, Ghasemi et al. [42] studied associations between *RsaI* polymorphisms of the prolactin gene and milk production traits in Montebeliard cows. They used PCR-RFLP method for identification of genotypes. They recorded genotypes frequencies of 0.81, 0.15 and 0.04 for A/A, A/B and B/B, respectively with frequency of 0.89 for A allele. The mean of their total milk yield was significantly higher in the AA genotype group compared to other groups where the mean of total milk production was 5805 litter for AA genotype, 4800 litter for AB genotype and 4835 litter for BB genotype. Wojdak-Maksymiec et al. [43] showed that three genotypes; AA (18.46%), BB (2.01%) and AB (79.53%) were shown in cattle and BB genotype had low milk

yield. Moreover, Uddin *et al.* [25] reported significant associations between PRL variants and milk production traits in dairy cattle.

There are different studies on prolactin gene *Rsa*I PCR/RFLP in buffalo that recorded monomorphic pattern AA of undigested fragment without genetic variation in all the studied population of Egyptian buffalo [29], Indigenous Anatolian Water Buffalo [27] and in Buffalo Population of Khuzestan-Iran [28].

In the present work, no significant differences between the two genotypes (AA and BB) and the studied semen parameters. On this respect, no previous studies were recorded in bulls.

It could be concluded that, BCS had no effect on buffalo bulls' semen quality. The presence of genetic polymorphism in prolactin XbaI locus of buffalo bulls may acts as a genetic marker using large number of animals.

Acknowledgements: This research was supported by National Research Centre, Egypt. Project No (11020101).

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(*Received* 8 / 4/ 2017;
accepted 6 / 8/ 2017)

تأثير حالة الجسم و التباين الوراثى فى PCR-RFLP لجين البرولاكتين على مواصفات السائل المنوى فى طلائق الجاموس

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تهدف هذه الدراسة الى تقييم تأثير حالة الجسم على مواصفات السائل المنوى فى الجاموس و تحديد التباين الوراثى لجين البرولاكتين كأساس للإختيار الطلائق ذات القيمة التربوية العالية. و قد تمت الدراسة على ستين من طلائق الجاموس المصرى يتراوح أعمارها ما بين 8-2 سنوات. و قسمت الحيوانات الى مجموعتين , الأولى ما بين اثنين و ثلاثة درجة BCSو تضم 33 حيوان و الثانية ما بين ثلاثة و اربعة درجة BCSو تضم 27 حيوان. تم تجميع السائل المنوى للحيوانات و تم فحصه مباشرة لكل من حجم القذفة و نسبة الحيوانات المنوية الحية و نسبة الحركة الفردية الطبيعية النشطة و أيضا نسبة سلامة الكروماتين. تم استخلاص الحمض النووى الديوكسى ريبوزى من عينات السائل المنوى لدراسة التركيب الجينى للبرولاكتين ياستخدام PCR-RFLP. و اثبتت النتائج ان حالة الجسم ليس لها تأثير على مواصفات السائل المنوى. و نتج عن استخدام تفاعل البلمرة المتسلسل لجين البرولاكتين قطعة طولها 678 نيوكليتيدة مزدوجة ثم استخدمت تقنية RFLP ياستخدام انزيم القطعXbaI . وتبين وجود اثنين من الطرز الجينى BB , AA و كان 91,67٪ من الحيوانات تمتلك نمط جينى AA بينما كانت نسبة 8,33٪ من الحيوانات تمتلك نمط جينى BB. و الخلاصة ان وجود تباين و اختلافات وراثية لجين اللبن البرولاكتين فى الجاموس المصرى يمكن استخدامه كأحد الدلالات الوراثية عند انتخاب افراد و سلالات جيدة للتربية.

الكلمات الدالة :الجاموس- السائل المنوي -تفاعل البلمرة المتسلسل ، RFLP -البرولاكتين .