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Effect of SNPs in Prolactin Promoter on Milk Traits in Egyptian Buffalo

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Abstract

Prolactin (PRL) plays a crucial role in the initiation and maintenance of lactation in animals. It is a potential quantitative trait locus and genetic marker of production traits in dairy cattle. However, no SNPs was detected in, and no association studies was conducted on, *PRL* gene in Egyptian buffalo. Therefore, this study aims to detect *PRL* gene polymorphisms and to study their effect on Egyptian water buffalo. A PCR product of 245 bp, including part of the promoter of *PRL* was amplified using PCR and subsequently, SSCP and nucleotide sequencing were conducted to identify its different allelic patterns in Egyptian buffaloes. Our results revealed presence of a novel T72C SNP in the PRL promoter with two genotypes (TC, CC). Chi-square test indicated presence of genetic disequilibrium with respect to studied variations and revealed significant difference in distribution of variants among buffaloes. Genetic diversity parameter showed moderate degree of polymorphism. The two genotypes (TC, CC) did not show association with milk traits (milk yield, fat%, protein%, and lactose%, solid %) in Egyptian buffaloes. We conclude that no significant difference in milk traits between Egyptian buffaloes have TC or CC genotypes and so breeders should not depend on this SNP (alone) in their selection for dairy animals.

Keywords: Prolactin; Egyptian buffalo; PCR; Sequencing; SNPs

Introduction

As a result to the actual increase in the world population and shortage of food supply, it becomes necessary for developing countries to maximize the production of their native animals to guarantee a sustainable source of food of animal origin. Among domestic animals, the water buffalo (*Bubalus bubalis*), particularly the river buffalo, which is the main buffalo breed in Egypt, holds great promise and potential for animal production. Indeed, the Egyptian buffalo plays a crucial role in Egypt economy by their livestock productivity from milk and meat. It contributes about 2.7% and 8.4% to the world buffalo's milk and meat, respectively [1]. The great importance of the Egyptian buffalo (*Bubalus bubalis*) and its superiority to the domesticated cattle is owed to their great adaptive capacity to the tropical climate, excellent nutritional benefits, resistance to the diseases and also the Egyptian consumers prefer buffalo milk to cattle milk because its white colour and good flavor. In addition, buffalo milk is characterized by high milk fat (7%), solid not fat (16%), lower levels of phospholipids and cholesterol and the fat has a higher proportion of saturated fatty acids, so that Egyptian buffalo consider the main dairy animal in Egypt [2].

To improve animal traits and productivity, the animals should be selected periodically on the basis of the genotype which will reflect on their productivity and on the national economics. There is a considerable interest in the application of molecular genetics technologies in the form of specific DNA markers that are associated with various productivity traits to promote more efficient and relatively easy selection and breeding of farm animals with an advantage for inheritable traits of meat and milk productivity. Many candidate genes have been identified and selected for analysis based on a known relationship with productivity traits [3-6]. The candidate genes that related to milk traits are called lactogenic genes. Among different candidates, the prolactin (PRL) gene seems to be promising, because it plays a crucial role in mammary gland development and in the initiation, maintenance of lactation and expression of milk protein genes [7]. Prolactin is a single chian polypeptide hormone secreted from specialized lactotroph cells of the anterior pituitary gland, furthermore, it is produced by numerous other cells and tissues, including the mammary gland [8,9]. The main functions of PRL are regulation of reproduction, promotion of lactation in mammals, synthesis of milk (lactogenesis) and maintenance of milk secretion (galactopoiesis) [10].

The bovine *PRL* gene was mapped on chromosome 23 [11] and consists of 5 exons and 4 introns with 10 kb in size [12,13], encoding a 229-amino-acid prolactin precursor. The signal peptide contains 30 amino acids; thus the mature bovine PRL is composed of 199 amino acids [14]. Several polymorphic sites have been detected within *PRL* gene and statistically significant associations between *PRL* variants and milk production traits have been described in dairy cattle. A previous study has reported the associations between polymorphisms in the coding regions of the bovine *PRL* and milk production traits [15]. Scanty researches have been conducted exploring the genetic diversity on molecular genetic basis in buffalo all over the world in comparison with other farm animals. Moreover, only very few studies were conducted on the Egyptian buffalo. Therefore, the aim of this study is to identify *PRL* SNPs in Egyptian water buffalo and to study their association with milk traits.

Materials and Methods

Sampling and DNA extraction

This study involved 200 pure Beheiry Egyptian water buffalo (*Bubalus bubalis*) kept on a farm located in Nucleus Herd, Nataff Gedeed station of MahaletMousa farm, Kafrelsheikh governorate. All records of milk production traits in Beheiry buffaloes were collected from farm records to be used for statistical analysis. Records covered the period from 2012 till 2014. Animals were kept under semi-open sheds. Lactating buffaloes were milked by hand twice daily at 7.00 a.m. and 5.00 p.m. throughout the lactation period, and milk production was recorded daily. Buffaloes were maintained under the same system of feeding in the farm. The animals were grazed on Egyptian clover (*Trifolium Alexandrinum*) during December to May with concentrate mixture and rice straw. While, during June to November, animals were fed on concentrate mixture, rice straw and limited amount of clover hay. The concentrate feed mixture was given twice daily before milking, while rice straw was offered once daily at 9.00 a. m, whereas clover hay or (silage) in the summer was offered at 11.00 a.m. Multi mineral licking blocks were available for animals in the stalls.

Blood samples were collected by jugular vein puncture into vacutainer tubes contain an anticoagulant (disodium EDTA) and kept in ice box then kept at -20 for further use. The genomic DNA was extracted using Gene JET genomic DNA extraction kit following the manufacturer protocol (Jena Bioscience, Jena/*Germany*).

Polymerase chain reaction (PCR)

A partial sequence of prolactin gene promoter was amplified by PCR using forward primer 5\- CAGCCAGCAATTTTGATGA-3\ and reverse primer 5\- GATGTTCATTTCTGGTCAGTATG-3\ designed by Primer 3.0 software based on the published sequences of Indian buffalo (GenBank accession number, AF426315). The PCR was carried out in a reaction volume of 50 µL, containing 4.0 µL DNA template (approximately 100 ng), 1.0 µL (0.20 mM) dNTP, 5.0 µL buffer, 3.0 µL (2.5 mM) $MgCl_2$, 2.0 µL 10 µmol/L forward primer, 2.0 µL 10 µmol/L reverse primer, 1.0 µL 10X Taq DNA polymerase (5 U/µL, Fermentas, #K1071, European Union), and 32 µL nuclease free water. Thermal cycling parameters were as follows: initial denaturation at 94°C for 5 min, 35 cycles of amplification (94°C for 40 s for DNA denaturation, annealing 56°C for 1 min, extension at 72°C for 1 min) and final extension at 72°C for 10 min. The samples were held at 4°C. PCR products (245bp) were resolved by electrophoresis on 2% agarose gel in 1X TBE, stained with ethidium bromide and visualized with UV light of gel documentation system (Biometra Biomedizinische Analytik, GmbH).

Single stranded conformational polymorphism (SSCP)

Five µL of PCR products were mixed with 5µL of denaturating dye (95% formamide, 25 mM EDTA, 0.025% xylene–cyanole and 0.025% bromophenol blue), heated in 95°C for 10min and chilled in ice then subjected in 12% PAGE (37.5:1 acrylamide: bis-acrylamide) in 1x TBE buffer at 200V for 6h at 4°C. The gel was stained with ethidium bromide as was previously described by [4].

DNA Sequencing

PCR products with expected size were purified using PCR purification kit following the manufacturer protocol (Jena Bioscience, # pp-201×s) to remove primer dimmers, primers, nucleotides, proteins, salt, agarose, ethidium bromide and other impurities. The purified PCR products were sent to MacroGen Company (South Korea) to sequence in both directions using ABI 3730XL DNA sequencer (Applied Biosystem, USA) and the identity of the sequenced PCR product was examined using Blast search against the published sequences of Indian buffalo on GenBank database. The alignments and assembly of the sequences were performed using Geneious 4.8.4 software.

Statistical Analysis

Allele and genotype frequencies were calculated by direct counting. Hardy–Weinberg equilibrium (HWE) assessed by the χ^2 test and population genetic indices, i.e. *He* (gene heterozygosity) and Ne (effective allele numbers; reciprocal of homozygosity) were computed by POPGENE software [16]. Polymorphism information content (PIC) was calculated and classified into low polymorphism (PIC value <0.25), median polymorphism (0.25<PIC value <0.5), and high polymorphism (PIC value >0.5) according to Botstein et al. [17]. GLM was used to analyse the relationship between variations of the PRL promoter and milk traits using software SAS 6.12 and the following model: $Y_{kljm}= \mu + A_k + G_l + D_j + E_{klm}$, where Y_{kljm} was the trait measured on each of the klm^{th} animals µ was the overall population mean A_k was fixed effect due to the k^{th} age (in days), G_l was the fixed effect associated with l^{th} genotype, D_j = covariate of the days in milking at the test day, and E_{kljm} was the random error. All data was described as least square means ± standard error of means (SEM). The significance of least square means was compared for significance using Duncan's test.

Results and Discussion

Detection of novel T72C SNP in *PRL* promoter

Previous studies have considered the bovine *PRL* gene as an excellent candidate for linkage analysis with quantitative trait loci (QTL) affecting milk production traits [18-20]. Allelic variation in the structural or regulatory sequences of the *PRL* gene would be of interest because of the possible direct and indirect effect on milk production. In the current study, a locus containing a partial sequence of buffalo *PRL* promoter determined by PCR was examined to search for any prospective polymorphisms (Figure 1).

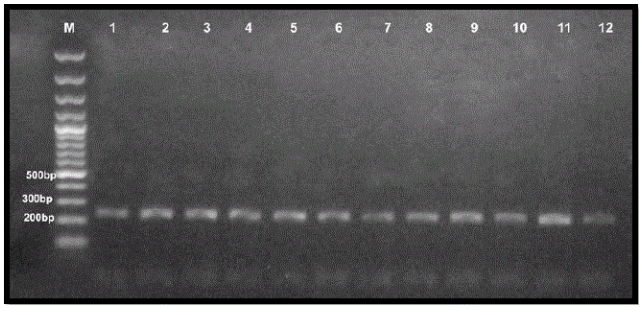

Figure 1: Ethidium bromide stained agarose gel of PCR products representing amplification of 245bp PRL locus (a part of the promoter) in 12 Egyptian buffaloes. M represents 100bp ladder.

Nucleotide sequences of this locus showed a novel T72C SNP (Figures 2A and 2B). In this SNP, the cytosine (C) nucleotide number 72 in *PRL* locus was replaced by thymine (T) nucleotide. SSCP patterns of *PRL* showed two different SSCP patterns; TC (three bands) and CC (one band) (Figure 3).

To our knowledge, this is the first association study applied on *PRL* gene of Egyptian buffalo. This SNP was novel and so was not overlapped with mutations reported in buffaloes or other animals. In agreement, some other SNPs were determined in the promoter regions of *PRL* in other animals: A1043G, A402G, A767C, G485T, C247A SNPs [19], and T175G and A446G SNPs [21] in cattle in addition to C499T SNP in pig [20].

Analysis of allele, genotype frequencies, Chi-Square (χ^2) and genetic indices

The genotype frequencies of T72C were 0.37 (74) for CC and 0.63 (126) for CT, with allele frequencies of 0.3150 for C and 0.6850 for T. Chi-Square (χ^2) equals 41.9 ($P<0.05$) among the 200 animals that were genotyped for this polymorphism. This means that all buffaloes were deviated from HWE. This deviation from HWE suggests a change in the distribution of alleles from one generation to the next.

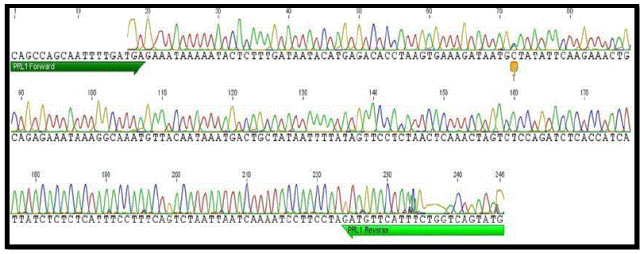

Figure 2A: Whole nucleotide sequences of PRL.1 locus showed T72C SNP (small colored box) in one Egyptian buffalo.

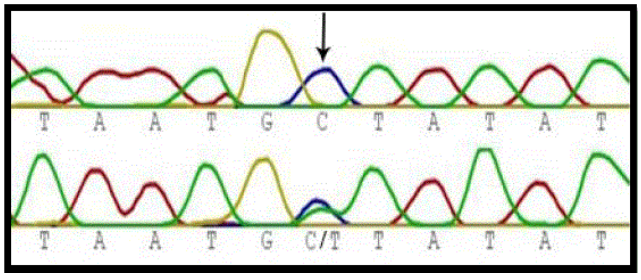

Figure 2B: Nucleotide sequences of PRL showed a T72C SNP in 2 Egyptian buffaloes. The arrow indicates the position of the SNP.

The value of the difference between expected and observed *He* was 0.431, while the value of *Ne* was 1.759. The PIC values were medium (0.338). The moderate *He* and *Ne* of T72C SNP shows the moderate polymorphism, moderate genetic diversity, moderate ability to maintain allelic stability during selection or mutation. Moreover, the moderate PIC (moderate polymorphism) is suitable as marker in molecular breeding [22] and indicates absence of genetic drift, no isolation or population substructure, or that they had not passed through artificial selections with a greater degree in history. This is in agreement with the results from Hardy-Weinberg which also show deviation from HWE.

Association between T72C SNP with milk trait

SNPs occurring within the *PRL* gene may influence the chemical composition of milk or at least be an effective DNA marker of a sub-region of dairy cattle genome [18]. Although the SNPs in this study were detected in non-coding regions, they may be associated with production traits as reported by previous studies [15,19,21]. One of these studies has identified two SNPs in bovine *PRL* promoter, A1043G and A402G that statistically associated with milk yield and fat content [15]. In addition, three other SNPs (A767C, G485T, and C247A) were identified within the promoter of the bovine *PRL* gene that significantly associated with milk yield, fat yield, and protein yield and protein percentage of dairy cattle [19]. Another two SNPs, T175G and A446G, also associated with milk traits were detected in the 5′UTR of the *PRL* promoter [21].

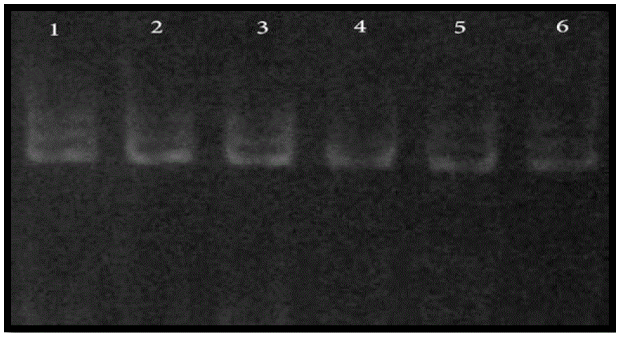

Figure 3: SSCP patterns of PRL (promoter) showed two different bands patterns: three bands pattern in TC genotype (lanes 1-3, 5, 6) and one band pattern in CC genotype (lane 4) in 6 Egyptian buffaloes.

Therefore, we used the GLM to study the statistical association between the T72C SNP and some milk traits; including milk yield, fat %, protein %, lactose % and solid %, in Egyptian dairy buffaloes. The results of this association analysis were detailed in Table 1. The T72C SNPs in *PRL* promoter showed no significant association with any of the examined milk traits ($P>0.05$).

Traits	TC	CC
Lactation length	259.35±6.43	261.63±6.14
Milk yield	2042.52±68.29	2063.62±58.31
Fat percentage	6.20±0.02	6.46±0.03
Protein percentage	4.21±0.03	4.10±0.03
Lactose percentage	5.45±02	5.19±02
Solid percentage	17.54±0.31	17.36±0.23

Table 1: Least squares mean (±SE) of different production traits for SSCP pattern of buffalo PRL. Lactation length is given in days and milk yield in Kg.

Although this SNP is not associated with milk traits, it may be linked with another SNP in the same gene or in another QTL related to milk traits. The phenotype effect of a single SNP may be affected by other SNPs in other loci in the same gene or other linked genes especially in polygenic traits such as milk traits. Thus, analyses of single SNP may be no effect on the phenotype, however when in combination, two SNPs or more displayed more profound impacts than in separation.

This agrees with the conclusion of that the inheritance of genotype combinations was more effective than that of a single SNP [23]. Therefore, further investigations including screening of the whole *PRL* gene sequences a long with statistical analysis of a large population size can shed the light on the significance of the detected SNP in this study in combination with other correlated SNPs and whether they associated with milk traits or not.

Conclusion

In this study, we detected a novel T72C SNP in the promoter region of the *PRL* gene, however, the statistical analysis showed no association between this SNP and some milk traits (milk yield, fat%, protein %, lactose % and solid %) in Egyptian dairy water buffaloes. Before excluding this SNP from breeding program for dairy buffaloes, it is necessary to extend our study to involve a large population size and whole sequences of the *PRL* gene. Considering the economic importance of the milk to the livestock industry, it appears clearly essential to further research on *PRL* in the buffalo.

Acknowledgments

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