IDENTIFICATION OF NOVEL SNP IN PROLACTIN PROMOTER AND ITS ASSOCIATION WITH MILK TRAITS IN BEHAIRY EGYPTIAN WATER BUFFALOES

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ABSTRACT

Prolactin hormone (PRL) plays a crucial role in the initiation and maintenance of lactation in the animals. It is a potential quantitative trait locus and genetic marker of production traits in dairy cattle. However, no SNP was detected and no association studies was conducted on PRL gene in Egyptian buffalo up till now. Therefore, the objective of the present study was planned to detect PRL gene polymorphisms in Behairy 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 Behairy Egyptian water buffaloes. Our results revealed the presence of a novel T72C SNP in the PRL promoter. Moreover, two genotypes (TC, CC) were identified using SSCP technique These genotypes did not show any association with milk traits (milk yield, fat%, protein%, lactose%, total solid%) in the animals under the study. We conclude that there were no significant [illegible] between TC and CC genotypes in [illegible] and hence the breeders should not depe[illegible] dairy animals

Keywords: Prolactin, Behairy Egyptian water buffaloes, 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 animal 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 live stock productivity from milk and meat. It contributes about 2.7% and 8.4% to the world buffalo's milk and meat, respectively ***(Abdel-Salam et al., 2010)***. Many candidate genes have been identified and selected for analysis based on a known relationship with productivity traits ***(Spelman and Bovenhuis, 1998).*** Among different candidates, the prolactin gene seems to be promising, because it plays a crucial role in mammary gland development and in the initiation and maintenance of lactation and expression of milk protein genes ***(Othman et al., 2011).*** 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 ***(Bole-Feysot et al., 1998; Perrot-Applanat et al., 1997)***. Prolactin involved in many endocrine activities and the main functions of PRL are regulation of reproduction, promotion of lactation in mammals, synthesis of milk (lactogenesis), and maintenance of milk secretion (galactopoiesis) ***(Freeman et al., 2000)***.

The bovine *PRL* gene was mapped on chromosome 23 ***(Hallerman et al., 1988)*** and consists of 5 exons and 4 introns with 10 kb in size ***(Cooke et al., 1981; Truong et al., 1984)***, 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 ***(Cao X et al., 2002).***

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 economically the genetic effects on milk production traits were evaluated ***(Lu et al., 2010)***. Because of limited researches have been conducted exploring the genetic diversity on molecular genetic basis in buffalo all over the world in comparison with other farm animals. Only very few studies were conducted on the Egyptian buffalo. Therefore, the aim of the present study is to identify *PRL* SNPs in Behairy Egyptian water buffaloes 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 Mehalet Mousa farm, Kafrelsheikh governorate. All records of milk production traits in Behairy buffaloes were collected from farm records to be used for statistical analysis. Records covered the period from 2012 till 2013.

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 from white blood cells using Gene JET genomic DNA extraction kit following the manufacturer protocol **(JenaBioscience, Jena /G*ermany*).** All equipments used in this study were autoclaved and sterilized befor collection of the samples as well as during all procedeurs in the laboratory.

Polymerase chain reaction (PCR):

A partial sequence of prolactin gene promoter was amplified by PCR using primers (see inserted table) designed by Primer 3.0 software based on the published sequences of Indian buffalo (GenBank accession number, AF426315).

Primer sequences, annealing temperature (Ta) and PCR products characteristics of part of the promoter of the *PRL* gene.

Gene	Forward primer ('5-'3)	Reverseprimer ('5-'3)	Ta (°c)	Size (bp)	Localization
PRL	CAGCCAGCAATTTTG ATGA	GATGTTCATTTCTGGTCAG TATG	56	245	Part of promotor

The PCR was carried out in a reaction volume of 50µL, containing 4.0µL DNA template (approximately 100ng), 1.0µL (0.20mM) dNTP, 5.0µL buffer, 3.0µL (2.5mM) $MgCl_2$, 2.0µL 10µmol/L forward primer, 2.0µL 10µmol/L reverse primer, 1.0µL 10x Taq DNA polymerase (5 U/µL, Fermentas, #K1071, European Union), and 32µLnuclease 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 were resolved by electrophoresis on 1.5% 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 at 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.

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 ***(El-Magd et al., 2013)***. 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 GenBank database of Indian buffalo (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignments and assembly of the sequences were performed using Geneious 4.8.4 software http://www.geneious.com/ web/geneious/home.

Statistiacl analysis:

- The Hardy–Weinberg (H–W) equilibrium was assessed by applying the $\chi 2$ test which was computed by POPGENE software (Version 3.1) ***(Yeh et al., 1999)***.
- The least square means estimates (LSM) with standard errors were calculated using software SPSS (Version 16.0) ***(Vasoo et al., 2009)***.

RESULTS

For detection of the *PRL*, genomic DNA from the blood samples of two hundreds Behairy Egyptian cow buffaloes were extracted. One locus containing a partial sequence of the promoter of *PRL* gene was chosen to search for any prospective polymorphisms. The quality of genomic DNA were checked on 1.5% agarose. The extracted DNA was used as a template for PCR to amplify a partial sequence of *PRL* the promoter with expected size 245 bp (**Fig. 1**).

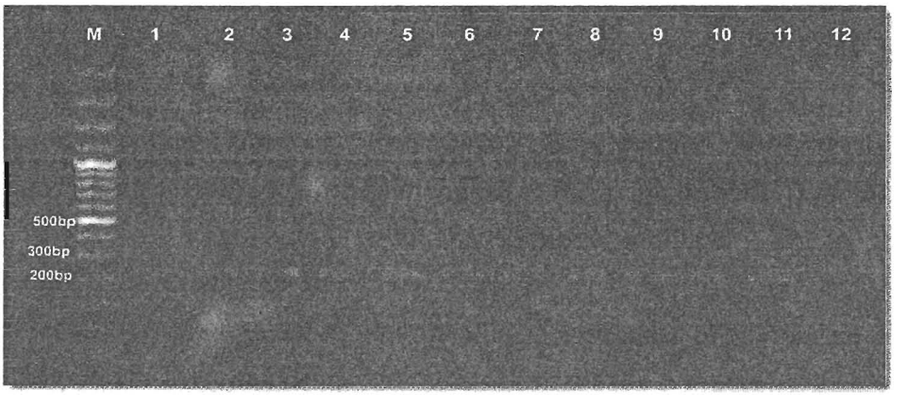

Fig. (1): Ethidium bromide stained agarose gel of PCR products representing amplification of 245bp *PRL* locus (a part of the *PRL* promoter) in 12 Egyptian buffaloes. M represents 100bp ladder.

PRL SNPs and their correlation with milk traits:

SSCP patterns of *PRL* showed dimorphic SSCP patterns (TC and CC), nucleotide sequences of *PRL* (the promoter) among Behairy Egyptian water buffaloes showed a novel T72C SNP. (**Fig. 2**).

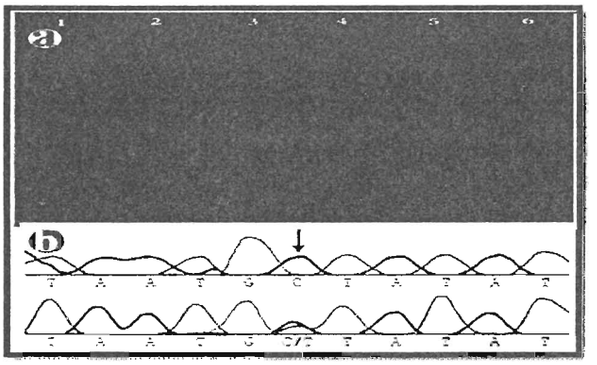

Fig. (2): (a) SSCP patterns of *PRL* (promoter) showed two different bands patterns in TC genotype (lanes 1-3) and 2 bands patterns in CC genotype (lanes 4-6) in 6 Behairy Egyptian water buffaloes. (b) synonymous T72C SNP was detected in *PRL* locus and the arrow indicate the position of the polymorphism.

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 41.900618 ($P < 0.05$) among the 200 animals that were genotyped for this polymorphism. This means that all buffaloes were deviated from HWE.

Table (1): Genotypic and allelic frequencies, value of χ^2test, and diversity parameter of T72C SNP of buffalo *PRL*.

PIC	*Ne*	*He*	P value	χ^2 (HWE)	Allele frequencies		Genotype frequencies (n)			SNP
					C	T	CC	TC	TT	
0.3384	1.7592	0.4315	0.000 (< 0.05)	41.900618	0.6850	0.3150	0.37 (74)	0.63 (126)	0 (0)	T72C

χ^2: Chi-Square value. HWE: Hardy-Weinberg equilibrium. *He*: Gene heterozygosity. *Ne*: Effective allele numbers, Polymorphic information content (PIC).

Least squares mean (LSM) for milk traits related to different SSCP variants obtained by statistical analysis were given in (Table 2). The T72C SNPs in *PRL.1* locus showed no significant association with milk traits ($P > 0.05$).

Table (2): 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.

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

DISCUSSION

The water buffalo holds great potential for animal production and plays a crucial role in Egypt economy by their livestock productivity of milk which is approximately 81 % of total milk production in Egypt ***(Ahmad et al., 2013)***. However, this animal remains underutilized and its production does not cover the local market requirements. Little efforts have been made to improve Egyptian buffalo genetic potentiality for milk production and they suffer from limited literature on genetic parameters of milk-related traits with no available reports on the use of genotype based selection to improve their milk production. Therefore, this study was aimed to apply genotype based selection on Egyptian buffaloes. We studied an important lactogenic gene prolactin (*PRL*) to look for any prospective SNPs and to study its association with milk production traits in Behairy Egyptian water buffaloes.

Allelic variation in the sequences of the *PRL* gene would be of interest because of the possible direct or indirect effect on milk production. SNPs occurring within the *PRL* gene may influence the yield and chemical composition of milk. Using PCR-SSCP and direct sequencing methods, we found one SNP, T72C in *PRL.1* locus in promoter region of the buffalo *PRL* gene. In this SNP, the cytosine (C) nucleotide number 72 in *PRL.1* locus was replaced by thymine (T) nucleotide. This SNP was novel and so it was not overlapped with mutations reported in buffaloes or other animals. The results of the sequencing of *PRL.1*was submitted to the GenBank database with accession number KC415283. In agreement, some other SNPs were determined in the promoter regions of *PRL* in other animals: A1043G, A402G, A767C, G485T, C247A SNPs ***(He et al., 2006)***, and T175G and A446G SNPs ***(Li et al., 2006)*** in cattle in addition to C499T SNP in pig ***(Korwin-Kossakowska et al., 2006).***

We have used SSCP method on a large number of animals. The results from SSCP confirmed the presence of T72C SNP as have been revealed by the presence of two different SSCP patterns; TC (with frequencies of 0.63) and CC (with frequencies of 0.37). The homozygous TT genotype has not observed in all genotyped animals (n=200). Absence of this genotype may be due to small size population (only 200 animals) or secondary to extensive selection within this population. The differences between genotypes are possibly due to long-term artificial insemination and selection towards high fat and protein contents of milk. Lack of TT genotype reflects on allele frequencies which become very higher (0.6850) for C allele and lower (0.3150) for T allele.

All examined buffaloes deviated from HWE ($P < 0.05$) for the T72C SNP. In general, deviation from the HWE proportions suggested that, at least one of the standard underlying assumptions for the test (non-overlapping generations, large population size with random mating, no mutation, no migration, and no selection) may be violated. Furthermore, HWE deviation means that, the genetic equilibrium in the populations under study was disturbed and the size of certain genotypes of the *PRL* was statistically different from the theoretically calculated size. This may be due to small sample size (only 200 animals).

Estimation of genetic diversity will throw light on the evolutionary pressure on the allele and the mutation the locus might have undergone over a time period. It is therefore important to calculate the gene diversity within/between the populations. There are some gene diversity parameters including, He (average heterozygosity), Ne (effective allele numbers) and PIC (polymorphic information content), which can be used to assess the diversity of a gene or DNA segment (locus) in a population. These parameters depend on the number of detectable alleles and the

distribution of their frequency. Values of 'He' parameter depend upon the type of marker system, i.e. whether the marker is co-dominant or dominant. Their range scores 0-1 in dominant markers system and 0-0.5 in co-dominant. In this study, He values were approaching 0.5 which indicates the presence of noticeable gene diversity. According to the classification of PIC (low polymorphism if PIC value < 0.25, moderate polymorphism if 0.25 < PIC value < 0.5, and high polymorphism if PIC value > 0.5) ***(Botstein et al., 1980)***, Egyptian buffaloes possessed moderate polymorphism at *PRL.1* locus. This reflected that there was a moderate genetic diversity within this locus in the analyzed animals.

Previous study reported the associations between polymorphisms in the promoter region of the bovine *PRL* gene and milk production traits in cattle by SSCP and sequencing techniques ***(Lu et al., 2010)***. This previous study identified two SNPs in *PRL* promoter, A1043G and A402G, that statistically associated with milk yield and fat content. In addition, three other SNPs (A767C, G485T, C247A) were identified within the promoter of the bovine *PRL* gene that significantly associated with milk yield, fat % and protein % of dairy cattle ***(He et al., 2006)***. Another two SNPs, T175G and A446G, also associated with milk traits were detected in the 5'UTR of the *PRL* promoter ***(Li et al., 2006).***

On the other hand, our study failed to find an association of T72C SNP with milk traits (including milk yield, fat%, protein %, lactose % and solid % in Egyptian dairy buffaloes (P> 0.05). This result could be explained by several reasons, e.g., the homozygous TT was not detected at *PRL1.1* locus; this fact may also influence the allelic frequency of the SNPs in the studied buffaloes. In the current study, T72C SNP does not change any of known binding sites for transcription factors as has revealed by the computer-aided (in silico) analysis using TESS program.

Therefore, it is likely that, T72C SNP may alter the expression of *PRL*. Therefore, the SNP occurring within *PRL* promoter does not influence milk traits and could not be used as an effective DNA marker in the dairy buffalo genome. Further studies on the candidate *PRL* gene are necessary to evaluate allele substitution effects and haplotype association studies in Egyptian buffaloes.

Further researches are needed to detect all possible SNPs in the full sequence of *PRL* gene in Egyptian buffaloes to find out the molecular basis for low milk productivity of our native buffaloes as compared to the foreign breeds.

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