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Association of β –Lactoglobulin Gene Polymorphism with Milk Yield, Fat and Protein in Holstein-Friesian Cattle

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ABSTRACT

Improving the efficiency of milk production and its constituents without increasing the size of the dairy herd is the foremost goal of the selection in dairy industry. The use of polymorphic genes as detectable molecular markers is a promising alternative to the current methods of trait selection once these genes are proven to be associated with traits of interest in animals. Beta-lactoglobulin (B-LG) is one of the most important genes that play a crucial role in the milk quality and coagulation process of cheese and butter. Identification of different B-LG genotypes and association with different milk performance traits in Egyptian Holstein cattle was performed through Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and DNA sequencing of two hundred blood samples. Digestion of 447bp PCR product with Hae III restriction enzyme revealed three genotypes (AA, AB, BB), with higher frequency of B allele (64%) than A allele (36%). Nucleotide sequence analysis of different genotypes revealed two point mutation at exon four, one of them (T301C) corresponding to the same amino acid asparagine and the other Single Nucleotide Polymorphism (SNP) (C390T) represented a non-synonymous mutation producing amino acid change of alanine to valine. Animals with genotype AA had more milk yield and protein % (11461kg and 3.45) respectively, while BB genotype recorded higher fat % (3.85). The information given in the present study will be extremely helpful for improving milk production traits in dairy cattle by marker-assisted selection and outlined a strategy to avoid long and costly traditional selection methods for dairy purposes in Holstein cattle

Key words: B-LG polymorphism, PCR- RFLP, Milk production, Holstein Friesian cattle

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INTRODUCTION

There is a significant enthusiasm for the utilization of molecular genetics technologies to recognize specific DNA markers that are connected with the economically important traits in order to make breeding program more effective through early selection of young animals as future breeding stock (Archana, 2013). The selection efficiency of complex quantitative traits in dairy cattle relies on the identification of candidate genes responsible for these traits and in addition the determination of causative DNA polymorphism in these genes. The genetic variability offers an open door for enhancement of cattle milk production through usage of genetic improvement programs (Abu Khaizaran, 2013).The candidate gene approach is a standout amongst the most vital ways to deal with quest for genetic markers related to production traits and in investigating polymorphism of structural and protein coding genes. At present, genetic markers research applied to animal breeding and production is focused mainly on analyzing mutations located within candidate genes and their relationship with economically important production traits (Oikonomou et al. 2011). Molecular technologies have been developed to recognize alleles and frequencies within milk protein genes, including specific PCR sequences, restriction enzymes and actually single nucleotide polymorphism (Ren et al., 2011).

Milk is an important source of essential nutrients for lactating calves and a key raw material for human food preparations (Reinhardt et al., 2012). Everywhere throughout the world, individuals satisfy around 13% of their protein requirement from milk and milk products. Milk proteins including casein and whey proteins have a crucial role in contributing to the nutritional qualities and properties of milk. The total milk protein composition unequivocally relies on the expression and secretion of individual proteins (Caroli et al., 2009). Beta-Lactoglobulin (B-LG) is the significant

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whey protein in the milk of cattle, sheep, dogs, and pigs but not found in humans, mice and other mammalian species. It could have a role in metabolism of phosphate in the mammary gland and the transport of retinol and fatty acids in the gut (Kusza et al., 2015). It has been mapped on bovine chromosome 11, spans 4.7Kb which is arranged in seven small exons, six introns and encodes 162 amino acid residues. Polymorphism of B-LG gene was firstly recognized in 1955 by Aschaffenberg and Drewry and a total of 15 alleles are known. Common alleles are A, B, C and D, with alleles A and B being the most incessant (Farrell et al., 2004). The bovine B-LG A allele differs from B allele by two amino acids substitution at positions 64 (aspartate → glycine) and 118 (valine → alanine) and also has a higher B-LG protein concentration than allele B. It is likely that this difference in amount of B-LG protein is not caused by the amino acid substitutions, but instead by different levels of expression of the corresponding A and B alleles of the B-LG gene (Hill, 1993). While, BB genotype is connected with higher casein and fat contents, which are ideal properties for cheese making additionally it might improve the quality of milk (Ren et al., 2011).

In light of critical role of B-LG gene in milk related traits and their genetic trends in dairy cattle, the point of this study was to identify B-LG gene polymorphisms and to determine the impacts of these variants on milk traits in Holstein Friesian cattle reared under Egyptian condition.

MATERIAL AND METHODS

Experimental and sample collection

This study was carried out on 200 randomly selected Holstein Friesian cattle from El-Shazly farm, Egypt. The phenotypic data including date of birth, date of calving, lactation, milk yield and lactation length were collected from daily farm records. The blood samples (5 ml) were collected under a sterile condition by jugular vein puncture into sterilized vacutainer tubes containing EDTA as an anticoagulant and then brought to the laboratory in ice box containing gel cool packs and stored at -20° C until DNA extraction.

Two hundred milk samples (10 ml) were collected during morning milking in order to represent the whole milking of each animal for detecting the milk constitution (fat, total protein ,solid not fat and lactose) by using ultrasonic portable milk analyzer (milko tester model- master mini).

DNA extraction

Genomic DNA extraction was performed with DNeasy Blood &Tissue Kit (QIAGEN, Germany) following the manufacturer's protocol. The quality and quantity of DNA was evaluated by 0.7 % agarose gel electrophoresis and by UV spectrophotometer, respectively.

PCR amplification and gel electrophoresis

Amplification of a 447bp fragment of B-LG gene covering intron III, exon IV and intron IV was done using a pair of forward (5′GCC TCA GAC TCA GTG GTGA 3′) and reverse (5′ACC ACA CAG CTG GTC TCC 3′) primers. The primers were designed using primer 3.0 software and the published nucleotide sequence of the Bos taurus B-LG gene (GenBank Accession No X14710.1). PCR reactions were done in a total volume of 25μL, consisting of 12.5µl master mix (Thermo Scientific, Fermentas), 2µl DNA template, 1µl of each primer (10pmol/µl) and deionized water up to 25µl. Amplification was carried out in a thermal cycler (Biometra, Germany) with the following conditions: initial denaturation at 94^{o}C for 2 min, followed by 35 cycles of 94^{o}C for 20 sec, 61^{o}C for 20 sec and 72^{o}C for 45sec with a final extension of 8 min at 72°C. The amplified fragments were separated on 1.5% agarose gel electrophoresis, imagined under UV transilluminator. The size of the amplified product was compared with the 100-bp ladder DNA marker.

PCR-RFLP analysis

The 447bp PCR products were digested by FastDigest Hae III restriction enzyme (Fermentas) at 37°C for 5 min. The digested products were resolved in 1.5% agarose gel containing ethidium bromide as the staining agent in 1×TAE buffer. A 100bp marker ladder was also run alongside the samples to ascertain the size of the amplified products. The digested products were visualized under UV light on a transilluminator and scored in the gel documentation system.

DNA sequencing

PCR products were purified with Gene JET PCR purification kit (Thermo Scientific Fermentas) following the manufacturer's guidelines and were straightforwardly sequenced utilizing both the forward and reverse primers of PCR amplification. The sequencing procedure was done by European Custom Sequencing Centre (GATC Biotech AG, Germany). The obtained sequences were edited manually utilizing Chromas Lite Ver. 2.01, (http://www.technelysium.com.au/chromas.html) and aligned with CLC Main Workbench 7 and Clustawl Omega software.

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Statistical analysis

The alleles and genotypes frequencies were estimated according to Falconer and Mackay (1996). The Pearson's chi-square ($\chi 2$) (P value < 0.001) was used to check whether the population is in Hardy-Weinberg Equilibrium (HWE) or not. Marker-trait association analysis was conducted by least square method of the General Linear Model (GLM) procedure of the statistical packages for social science using the following linear model.

$Yij=\mu+Gi+eij$

Where, Yij= Observation of the target trait, μ=Overall mean, Gi =Fixed effect of ith genotype, eij =Random error.

Duncan's multiple range tests had been used for comparing the means. Additive effects (allele substitution effect) were estimated through adding an additional regression covariate with value 0, 1 and 2 to account for number of AA, AB and BB genotypes, respectively.

RESULTS AND DISCUSSION

The study of candidate genesis one of the vital procedures to figure out if particular genes are connected with the economic important traits in farm animals. In marker assisted selection of dairy cattle, some genes are proposed as potential candidates associated with milk performance traits. Among the different candidates, B-LG gene that influences the milk production parameters and quality of milk protein. Their polymorphisms mostly clarify the genetic variance and enhance the estimation of breeding value.

B-LG gene fragment was successfully amplified using polymerase chain reaction technique for all samples and resulted in a single product of 447bp (Figure 1A). Digestion of this fragment with Hae III restriction enzyme revealed three genotypes which are designated as AA (236, 90, 74 and 47 bp), AB (310, 236, 90, and 74 and 47 bp) and BB (310, 90 and 47 bp) (Figure 1B). PCR products representing different genotypes were sequenced and two sequences revealing A and B alleles were submitted to the GenBank and deposited under accession numbers KR732930 and KR732929, respectively. The nucleotide sequence analyses revealed the presence of two SNPs in exon four (T301C and C390T) and two in the intronic region (T226C and C276T) of B-LG gene (Figure 2). The T301C SNP was silent base substitutions corresponding to asparagine amino acid (aa) and the other C390T SNP was a non synonymous mutation leading to substitution of alanine by valine. These results were similar to that reported by Getachew (2010), Piatkowska et al. (2011), Hristov et al. (2013) and Mir et al. (2014) in Holstein cattle.

The genotype frequencies were 0.50 (100) for BB, 0.22 (44) for AA and 0.28 (56) for AB with allele frequencies of 0.36 for A and 0.64 for B among animals that were genotyped for this polymorphism. Chi-Square ($\chi 2$) value was 30.78 (P value=0.001). This means that the genotype distributions within cattle population deviated from HWE (P<0.05). These data are in the context with Ren et al. (2011) and Singh et al. (2014) who reported the highest frequency of allele B and BB genotype in Holstein cattle.

The polymorphism of B-LG gene was studied in other cattle breeds such as Girolando cows (Botaro et al., 2008),Turkish cattle breeds (Dinc et al., 2013), Sahiwal cattle (Mir et al., 2014 and Kishore et al., 2014), and Mexican Jersey cattle (Batista et al., 2015), they found that BB genotype and B allele were the most frequent in all studied breeds. In contrast to these findings Ivanković et al. (2011) and Lukac et al. (2013) reported the predominance of AB genotype in Estonian dairy cattle and Holstein cattle respectively. This can be explained by different history of the breeds, long-term geographical isolation and selection towards high fat and protein percent of milk in some breeds such as Holstein Frisian cows.

Association of AA, AB, and BB genotypes of B-LG gene with the milk traits were analyzed in Holstein Frisian cattle. The results indicated that animals with AA genotype showed higher milk yield, protein % and solid % (11461 kg, 3.45% and 9.27%) respectively. The differences were more obvious for the fat % as animals with BB genotype were higher (3.85%) than that of AA and AB genotypes (2.94% and 2.97%) respectively and there is no significant (P<0.05) effect on lactose % (Table 1). Similar effect of B-LG genotypes had been observed by Heidri et al. (2009), Getachew,(2010) and Piątkowska et al. (2011) they noted the highest milk yield for cows with AA genotype, whereas milk of B-LG BB cows exceeded milk obtained from cows with AA and AB genotypes as regards fat %.

In contrast Ahmadi et al. (2008) reported strong association between BB genotype and protein % while there was no association between B-LG genotypes and milk yield or milk fat %. Mir et al. (2014) found no differences in milk compositional characteristics among different genetic variants of B-LG in Holstein, Girolando, Czech Fleckvieh and Sahiwal cattle breeds. Hirstov et al. (2013) showed that the BB genotype determines higher milk production, Singh et al. (2014) found that AB and BB genotypes of B-LG had a significant (P< 0.05) effect on total milk yield and peak yield compared with AA.

Substituting the A allele with the B allele decreased the milk yield, protein % and solid % with (4503.7kg, 0.271 %, 0.318 %) respectively this demonstrates the superiority of the A allele with respect to quantitative milk traits but substituting the A allele with the B allele increased the percentage of milk fat by 0.525 % (Table 1).

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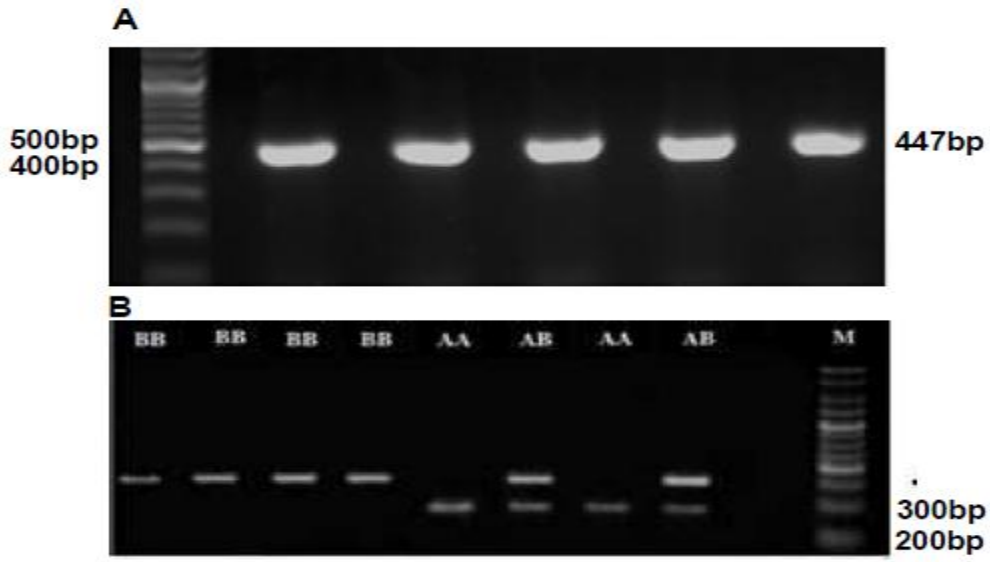

Figure 1. **A**: PCR amplification of β-lactoglobulin gene in Holstein Frisian cattle. **B:** Restriction fragment patterns of β-lactoglobulin gene after digesting with Hae III. M: 100bp ladder

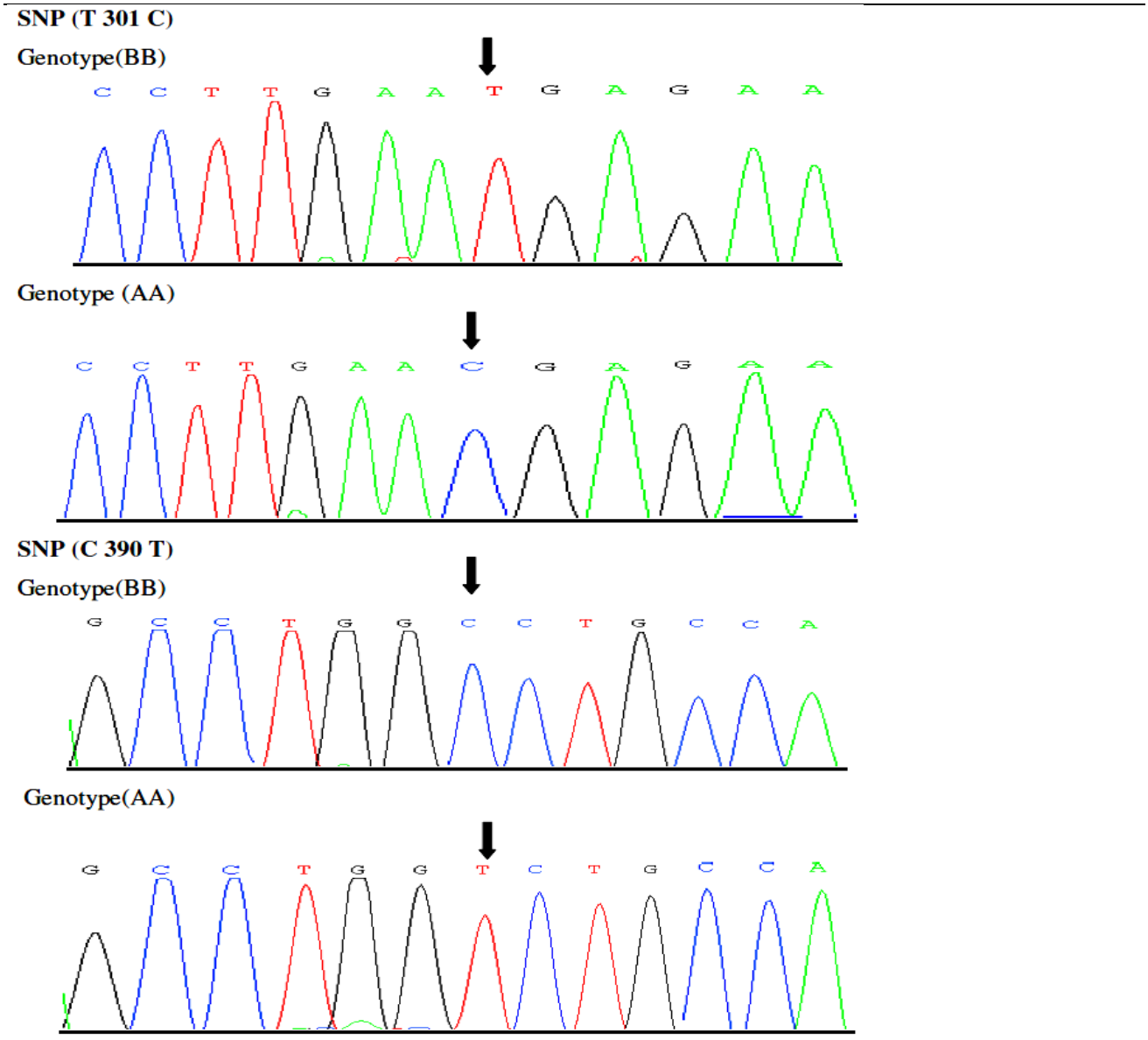

Figure 2. Relative sequenced peaks of β-lactoglobulin genotypes AA and BB in Holstein Frisian cattle. Arrows refers to site of base change

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Table 1. Least square means ± SE for milk production traits with different B-LG genotypes and allele substitution effect in Holstein Frisian cattle

Trait	Genotype (mean ± SE)				Allel substitution effect (B allel)
	AA	AB	BB	F-value	
Milk yield	11461 ± 494[a]	8475 ± 675[b]	2784 ± 176[c]	122.10[*]	- 4503.7[*]
Fat %	2.94 ± 0.122[b]	2.97 ± 0.082[b]	3.85 ± 0.045[a]	64.03[*]	0.525[*]
Protein %	3.45 ± 0.092[a]	2.86 ± 0.117[b]	2.87 ± 0.071[b]	11.39[*]	0.271[*]-
Lactose %	4.12 ± 0.033[a]	4.11 ± 0.059[a]	4.17 ± 0.034[a]	0.688[NS]	0.029
SNF %	9.27 ± 0.111[a]	9.13 ± 0.092[a]	8.60 ± 0.051[b]	25.11[*]	- 0.318[*]

* = Significant at (P< 0.05); NS= non-significant; SNF= solid not fat

CONCLUSION

In the present study PCR amplification and RFLP analysis were found to be a fast and sensitive method to recognize B-LG genotypes directly at the DNA level. It may be stated that Holstein Friesian cattle breed was polymorphic for B-LG gene producing 2 types of alleles and 3 types of genotypes. The frequency of B allele was comparatively higher than A. The genotype AA produced significantly higher milk yield, protein % whereas genotype BB yielded higher Fat % in Holstein Friesian cattle. Hence, B-LG genotyping can be used in selecting superior genetic structures for milk production in young females in shorter time than the traditional selection could. The selection of these superior individuals in early age and culling of the lower ones based on their genotype could take an interest in enhancing milk production of animals.

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Competing interests

The authors have declared that no competing interest exists.

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