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Investigation Of STAT5A, OLR1, LF And TLR4 Variants And Its Association With Milk Yield In Egyptian Buffalo.

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ABSTRACT

In this work, we selected 19 SNPs that are located in 4 genes, *STAT5A*, *OLR1*, LF and TLR4, because of their contribution and association with milk production traits in cattle. The selected SNPs were genotyped on 74 multiparous Egyptian buffaloes via sequenom assay. Only 7 SNPs showed polymorphic patterns, one of them is located in intron 1 of OLR1 gene at nucleotide position 423. This SNP found to have a minor allele frequency (MAF) of 0.14. The other one lies in intron 1 of TLR4 at nucleotide position 4525, its MAF is 0.025. The remaining 5 SNPs are located in LF gene at nucleotide positions -190, -472, -292, -19 and +35 from the transcription start site. Their MAF are: 0.13, 0.19, 0.19, 0.18 and 0.19 respectively. None of the polymorphic SNPs showed significant associations with milk yield of Egyptian buffalo what could be due to small sample size. Although no significant associations were detected between any of the investigated SNPs and milk yield in the current study, this study shades a light on some polymorphic sites in Egyptian buffalo that could be used as genetic markers. Hence, further association studies on these polymorphic sites in a larger sample size and against different traits are recommended.
Keywords: Buffalo, milk yield, STAT5A ,OLR1, LF, TLR4, Sequenom assay.

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INTRODUCTION

Buffalo is regarded as an economically essential livestock species especially in third world countries. It plays a crucial role through contributions in cultural and social aspects [1]. In Egypt, Water buffalo contributes 81% of the annual milk production representing the chief milk's producers [2]. The Egyptian consumers prefer buffalo's milk due to its white color, flavor and high fat content [3]. Milk yield produced by dairy buffalo ranges from 1200 to 2100 Kg per lactation and the lactation length is about 210 to 280 days [2].

Several genes were reported to be associated with milk production traits in cattle, of these: Signal transducer and activator of transcription 5A (STAT5A) [4-6], Oxidized low-density lipoprotein receptor 1 (OLR1) [7, 8], Lactoferrin (LF) [9] and Toll-like receptor 4 (TLR4) [10].

STAT5A is a transcription factor that activates the action of some hormones such as Prolactin, Growth Hormone and Caseins [11]. Prolactin hormone signalling leads to STAT5 activation which translocate inside the nucleus, which in turn leads to activation of the milk protein genes transcription [12].

OLR1 is the major protein which is responsible for binding, internalizing and degradation of oxidized low-density lipoprotein (OLDL) [13]. Khatib, Leonard [7] identified OLR1 as a functional candidate gene for milk fat profile. Lately, Schennink, Bovenhuis [8] reported a QTL for long chain fatty acids in oxidized low density OLR1 gene on BTA5 in Dutch Holstein population.

LF is called the red protein which is an iron binding glycoprotein of 80 kDa. It is related to family of serum transferrin, it is found in several exocrine secretions such as cervical mucus, semen, saliva and tears and also it is present in the milk [14]. Its locus on chromosome BTA22 was predicted to harbor several QTLs for protein yield **(PY),** protein percentage **(%P)** and Somatic Cell Score **(SCS)** and milking speed [15-18].

Toll-like receptors are membrane integral proteins mainly expressed in macrophages and dendritic cells [19]. They are considered the first line of defense against pathogens by recognizing of specific pathogen associated molecular patterns (PAMPs) [20]. Recently, several immune related genes were identified as potential candidates for milk production traits in dairy cattle [21-24]. Moreover, Li, Zhang [10] reported association of TLR4 gene with milk performance.

With the aim of developing a single test to identify and manage the natural variation in milk yield trait of Egyptian buffalo, we have selected the aforementioned genes to study the association of their genetic variants with milk yield.

MATERIALS AND METHODS

Blood Sampling and DNA isolation:

A total of 74 Egyptian Buffaloes were used in the current study. These animals were born between 1995 and 2007 in both Gemmeza station which located in El-Gharbiya governorate, and from another private station which is located in El-Beheira governorate. The blood samples were collected under supervision of local veterinarians. About 10 ml blood was collected from the jugular vein of each experimental animal in vacutainers containing Na2EDTA as an anticoagulant. Genomic DNA was extracted from whole blood with phenol chloroform conventional method according to Sambrook and Russell [25], then kept at -20°C. The DNA concentration was measured on UV spectrophotometer (Jenway, 6705, Bibby scientific Ltd, Essex, UK) at 260 nm and 280 nm then diluted to 50 ng μl^{-1}.

Phenotype

Records from daily milk yield were used in this study. A number of editing steps were performed to ensure a homogeneous data set. The editing process was carried out to exclude records from the sixth and later lactations. Other criteria were set to include only records between 5 and 290 days in milk and lactations with a minimum of 150 days in milk. Before data editing, the number of daily milk records were 47,643 records. After editing, a total number of 74 buffalo with 41,226 records were used.

Genotyping:

Based on previous study a total of 19 SNPs located in 4 candidate genes (STAT5A, OLR1, LF and TLR4) (Table 1) were selected to investigate their association with milk yield in Egyptian buffalo.

Table 1: The selected genotyped SNPs and their positions

SNP ID	Type	Location in the gene	Scaffold position	Relevant animal	Reference
STAT5A-1 STAT5A-2 STAT5A-3 STAT5A-4	CCT deletion	12549 (Intron 15)	(700429)	Polish cattle	Flisikowski and Zwierzchowski [26]
	C/T substitution	6854 (Exon 7)	(706147)	Italian Brown Swiss cattle	Selvaggi, Dario [27]
	A/G substitution	9501 (Intron 9)	(703480)	Black and White cattle	Brym, Kaminski [28]
	T/C substitution	12743 (Exon 16)	(700235)	Polish cattle	Flisikowski, Strzalkowska [29]
OLR1-1 OLR1-2 OLR1-3 OLR1-4	A/C substitution	8232 (3'UTR)	(379396)	Italian Brown Swiss cattle	Khatib, Leonard [7]
	A/G substitution	423 (Intron 1)	(389445)	Indian buffalo	Shabir, Jawale [30]
	A/T substitution	866 (Intron 1)	(389002)	Indian buffalo	Shabir, Jawale [30]
	T/C substitution	843 (Intron 1)	(389025)	Indian buffalo	Shabir, Jawale [30]
LF-1 LF-2 LF-3 LF4 LF-5 LF-6 LF-7 LF-8	C/T substitution	-586 (Promoter)	(414042)	Holstein-Friesian cattle	O'Halloran, Bahar [31]
	A/G substitution	-190 (Promoter)		Holstein-Friesian cattle	O'Halloran, Bahar [31]
	A/C substitution	-28 (Promoter)	(413486)	Holstein-Friesian cattle	O'Halloran, Bahar [31]
	A/G substitution	-472 (Promoter)	(413930)	Indian buffalo	Kathiravan, Kataria [32]
	G/C substitution	-209 (Promoter)	(413667)	Indian buffalo	Kathiravan, Kataria [32]
	G/C substitution	-292 (Promoter)	(413750)	Indian buffalo	Kathiravan, Kataria [32]
	T/C substitution	-19 (Promoter)	(413477)	Indian buffalo	Kathiravan, Kataria [32]
	T/C substitution	+35 (5'UTR)	(413424)	Indian buffalo	Kathiravan, Kataria [32]
TLR4 -1 TLR4 -2 TLR4 -3	C/G substitution	-226 (Promoter)	(54698)	Chinese Holstein cattle	Sharma, Leyva [33]
	A/G substitution	4525 (Intron 1)	(49856)	Chinese Holstein cattle	Wang, Xu [34]
	C/G substitution	8664 (Exon 3)	(45167)	Canadian Holstein bull cattle	Wang, Xu [35]

SNPs position based on water buffalo assembly 2.0 UMD + CASPUR (Scaffold, STAT5A: NW_005784710.1, OLR1: NW_005784939.1 , LF: NW_005785445.1 and TLR4: NW_005784801.1).

A total of 74 buffalo were genotyped for the selected 19 SNPs using Sequenom iPLEX Gold on the Sequenom platform offered by the Neogen genotyping service (www.neogen.com, USA). MassARRAY®Design software (www.sequenome.com) was used for designing both PCR and Mass EXTEND® primers for the multiplexed assay.

Association analysis

Frequency estimations of each SNP alleles and genotypes were calculated by direct counting. GenAlEx 6.5 software [36]; was used to calculate the deviation from Hardy-Weinberg proportion.

To test the association between daily milk yield and genotypes, the following model was applied using mixed model in SAS, version 9.4 (SAS 2014, SAS Institute Inc., Cary, NC, USA). In this model, the first order autoregressive covariance structure for repeated statement was used:

$$Y_{ijklm} = \mu + HYS_i + Lac_j + b_{k1} (DIM) + b_{k2} [exp (-0.05*DIM)] + g_l + \varepsilon_{ijklm}$$

where Y_{ijklm} is daily milk yield; μ is the overall mean of observations; HYS_i is the fixed combined effect of herd (h), year (y), and season (m) of calving, where seasons were defined as calendar quarters: January to March, April to June, July to September, and October to December; Lac_j is the fixed effect of lactation number; b_{k1} and b_{k2} are two regression coefficient associated with the fixed lactation function, where DIM is days in milk [37]; g_l is the random effect of SNP genotypes; and ε_{ijklm} is the residual error. Post hoc, differences among genotype classes were tested for significance using a Tukey–Kramer test as implemented in SAS (SAS 2014, SAS Institute Inc., Cary, NC, USA).

RESULTS

In this Study we selected 19 SNPs located in STAT5A, LF, OLR1 and TLR4 to investigate their association with milk yield in Egyptian buffalo. These SNPs were genotyped in 75 buffalo, the allele and genotype frequencies are represented in table2.

Genotyping results showed that, none of the chosen 4 SNPs of STAT5A show polymorphisms in any of the genotyped animals. The only observed allele for STAT5A-1 is the insertion of CCT, while the only observed allele for STAT5A-2, STAT5A-3 and STAT5A-4 are C, G and T respectively.

In contrast, only SNP OLR1-2 on OLR1 gene (A/G substitution in intron 1) showed a polymorphic pattern. A allele found to be the most frequent in the genotyped buffalo with frequency of 0.88 The Genotype AA is the most frequent with frequency of 0.75, while heterozygous AG frequency is 0.24. On the other hand, genotype GG not presents. While the only observed allele for OLR1-1, OLR1-3 and OLR1-4 are G, A and A respectively.

In lactoferrin gene, 3 SNPs were monomorphic, 2 of them were previously reported in cattle (LF-1 and LF-3), and the last one was detected in Indian buffalo (LF-5). The only observed allele for LF-1, LF-3 and LF-5 are C, C and G. The remaining five SNPs showed polymorphic patterns, these are:

SNP LF-2 (A/G substitution in position -190 in promoter) showed G allele with frequency 0.87. Genotype GG found to be the most frequent with frequency of 0.77, while heterozygous AG frequency is 0.19. On the other hand, genotype AA is the least frequent (0.34).

SNP LF-4 (A/G substitution in position -472 in promoter) showed the highest frequency (0.81) for allele A. Genotype AA is the most frequent with frequency of 0.68, while heterozygous AG frequency is 0.26. On the other hand, genotype GG is the least frequent (0.55).
The G allele of SNP LF-6 (G/C substitution in position -292 in promoter) showed the highest frequency (0.82). Similarly, the genotype GG is the most frequent with frequency of 0.68, while heterozygous GC frequency is 0.26. On the other hand, genotype CC is the least frequent (0.55).

SNP LF-7 (T/C substitution in position -19 in promoter) showed the highest frequency for T allele (0.82). Genotype TT is the most frequent with frequency of 0.68, while heterozygous TC frequency is 0.27. On the other hand, genotype CC is the least frequent (0.41).

The T allele of SNP LF-8 (T/C substitution in position +35 in 5' untranslated region) is the most frequent with frequency of 0.82. Genotype TT is the most frequent with frequency 0.68, while heterozygous TC and CC frequencies are 0.26 and 0.55 respectively.

TLR4 showed only one polymorphic SNP (SNP TLR4_2: A/G substitution in intron 1) where its G allele is the most frequent with frequency of 0.98. Similarly, Genotype GG is the most frequent with frequency of 0.96, while heterozygous AG frequency is 0.4. On the other hand, genotype AA was absent. This SNP was excluded from association analysis due to low MAF. While the only observed allele for both TLR4-1 and TLR4-3 is C.

None of the genotyped SNPs deviated from the Hardy-Weinberg proportion.

Association analysis was performed to evaluate the association between the polymorphic SNPs and milk yield in Egyptian buffalo; however, no significant associations were detected between any of the examined SNPs and milk yield (table 2).

Table 2: Alleles, genotypes frequencies and association analysis of the polymorphic SNPs in OLR1, LF and TLR4 genes with milk yield in Egyptian buffalo.

SNP ID	Alleles		Genotype (F)	LSM±SE	P-value
	A1 (F)	A2 (F)			
OLR1_2	A (0.88)	G (0.12)	AA (0.754)	8.94±0.36	0.5744
			AG (0.246)	9.25±0.38	
			GG (0.000)	-----	
LF2	G (0.87)	A (0.13)	AA (0.034)	9.39±1.13	0.5685
			AG (0.190)	9.30±0.56	
			GG (0.776)	9.86±0.31	
LF4	A (0.81)	G (0.19)	AA (0.685)	8.85±0.26	0.3379
			AG (0.260)	9.16±0.38	
			GG (0.055)	7.80±0.91	
LF6	G (0.82)	C (0.18)	CC (0.055)	7.78±0.91	0.3071
			CG (0.260)	9.17±0.39	
			GG (0.685)	8.81±0.26	
LF7	T (0.82)	C (0.18)	CC (0.041)	7.80±0.91	0.3379
			CT (0.274)	9.16±0.38	
			TT (0.685)	8.85±0.26	
LF8	T (0.82)	C (0.18)	CC (0.055)	7.81±0.90	0.2717
			CT (0.260)	9.24±0.38	
			TT (0.685)	8.84±0.26	

A1: major allele, A2: minor allele, F: frequency, LSM: least square means, SE: standard error.

DISCUSSION

Since the dawn of history, phenotypic data or performance data were used for animal breeding programs. That was insufficient to analyze genes responsible for expressing quantitative traits [38]. Applying genetic markers using new technologies enabled scientists to improve the efficiency and accuracy of animal breeding programs. Marker assisted selection (MAS) is the cornerstone for efficient program as it combines the information of genetic molecular polymorphisms (marker loci) with data on phenotypic variation among individuals [39]. The majority of desirable traits are polygenic and the first step in the determination of the powerful genetic combination is searching for so called candidate genes with an impact on these traits (Dekkers, 2003). Based on the fact that both buffalo and cattle are in a close evolutionary relationship [40], we selected some genes which were previously reported to be associated with milk production trait in cattle to investigate the association of their nucleotide variants with milk yield in Egyptian buffalo.

Variants on STAT5A were widely studied and reported to be associated with different milk production traits in different cattle breeds [4, 29, 41]. In the current study we selected 4 SNPs in STAT5A to study their association with milk yield in Egyptian buffalo. However, none of the selected SNPs in this gene found to be polymorphic in the investigated animals what suggests their absence in the Egyptian buffalo. This observation could be referred to the natural difference between cattle and buffalo.

Although three of the selected SNPs on OLR1 were detected previously in Indian buffalo by Shabir, Jawale [30], two of them are monomorphic in Egyptian buffalo, what could suggest that these 2 SNPs (OLR1-3 and OLR1-4) are specific to Indian breed. The only observed polymorphic SNP (OLR1-2) showed no significant association with milk yield. This could be due to the small sample size or this SNP could be in association with other trait. SNP OLR1-1 was widely studied in different cattle breeds [7, 8] and reported to be in association with milk performance. However, this SNP is monomorphic in our studied populations of Egyptian buffalo which in consents with a previous study on Mehsana buffalo breed by Deshpande, Rank [42] who reported a monomorphic pattern of the same SNP. This finding suggests that this SNP could be specific to cattle. However, Hassanane, Neama [43] reported this SNP in Egyptian buffalo. This conflict in the obtained results may be due to different methods sensitivity as we used Sequenom assay for SNPs genotyping which is more accurate than the restriction fragment length polymorphism (RFLP) that used by Hassanane, Neama [43].

Lactoferrin gene has been assigned to bovine chromosome 22, as it consists of 17 exons [44]. The locus of this gene is known to harbor several QTLs for protein yield, SCS and milking speed [16, 18]. In this study, we selected 8 SNPs in LF gene, 3 of them were reported previously in cattle, these SNPs are: SNP (LF-1) which was detected in position -586 from the transcription start site [31], SNP (LF-2) which was localized in position -190 from the transcription start site [31] and SNP (LF-3) which lies in position -28 from the transcription start site upward TATA box region [31]. The remained 5 SNPs were previously detected in Indian buffalo [32] (LF-4, LF-5, LF-6, LF-7 and LF-8) these SNPs were localized in important positions near from transcription start site.

Genotyping the 8 SNPs in Egyptian buffalo showed that 3 SNPs were monomorphic. Two of them were previously detected in cattle (LF1 and LF-3), that could be due to species differences. The Other monomorphic SNP were previously detected in Indian buffalo (LF-5), which could be due to difference between breeds. The remained 5 SNPs were polymorphic, these are: SNP LF-2, which showed G allele as the most frequent. This observation is in line with O'Halloran, Berry [45] who studied this SNP and reported G allele as the most frequent allele in Holstein Friesian sires.

SNP LF-4, which lies in position -472 from the transcription start site. Its G allele was reported as the most frequent in Indian buffalo by Kathiravan, Kataria [32]. In contrast our study on Egyptian buffalo revealed that A allele is the most frequent.

The G allele of SNP LF-6 that locates in position -292 from the transcription start site is the most frequent in Egyptian buffalo. This observation is in line with Kathiravan, Kataria [32] who reported G allele as the most frequent in Indian buffalo.

SNP LF-7, which locates in position -19 from the transcription start site immediately downstream to open promoter complex. The T allele of this SNP was the most frequent in Egyptian buffalo. This result is in line to the study by Kathiravan, Kataria [32] who reported T allele as the most frequent in Indian buffalo.

SNP LF-8 that locates in 5'UTR in position +35 from the transcription start site immediately upstream the transcription start site. The T allele of this SNP was the most frequent in Egyptian buffalo. This result is in line with Kathiravan, Kataria [32] study who reported T allele as the most frequent in Indian buffalo too.

Previous studies have reported association between several SNPs on TLR4 and milk production trait in cattle. In this study we selected three SNPs on TLR4 based on their effects on milk production. SNP TLR4-1 is present in exon 3 and lead to substitution of amino acid Threonine to Isoleucine, it was first described by Sharma, Leyva [33], who reported an association between this SNP and expected breeding values for lactation persistency and SCS in Canadian Holstein bull population. Furthermore, Beecher, Daly [21] reported an association between this SNP and milk fat and protein percentage in late lactation in Holstein-Friesian, Jersey, Norwegian red, Montbeliarde and Holstein-Friesian * Jersey cows but not in Holstein-Friesian bulls. Recently, Li, Zhang [10] reported a significant association between this SNP and milk production traits in Chinese Holstein cows. SNP TLR4-2 was detected in intron 1 by Wang, Xu [34] in Chinese Holstein cows and showed an association with SCS. SNP (TLR4-3) locates in the 5' untranslated region (5'UTR) which was first described by Sharma, Leyva [33] who identified this SNP at position -226 in the putative promoter region and reported a significant association between this SNP and expected breeding values for lactation persistency and somatic cell score in Canadian Holstein bull population.

Genotyping of these 3 SNPs in Egyptian Buffalo showed that two SNPs (TLR4-1 and TLR4-3) were monomorphic which could be due to difference between species. The only polymorphic SNP (TLR4-2) in Egyptian buffalo showed the lowest frequency for allele A (0.02). This result is in contrast to the result on cattle by Wang, Xu [34] where A allele had the highest frequency. However, none of the polymorphic SNPs in our study showed significant association with milk yield in Egyptian buffalo which could be due to low sample size or these SNPs might be associated with other traits.

CONCLUSION

In this work we studied 19 SNPs that located in STAT5A, OLRI, LF and TLR4. Genotyping of these SNPs in Egyptian buffalo showed that only 7 SNPs were polymorphic, while the other SNPs were monomorphic, what could be referred to the differences between species and/or breeds. Although no associations between the investigated SNPs and milk yield were detected, this study shades a light on important polymorphic sites in Egyptian buffalo which could be associated with milk performance or any different traits. Hence, further association study on these polymorphic SNPs using a larger sample size and against different traits is recommended.

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