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Analysis of selected milk traits in Palestinian Holstein-Friesian cattle in relation to genetic polymorphism

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Modern dairy cattle breeding strategies depend on linkage analysis and quantitative trait loci (QTL) of genes involved in milk yield and composition. This is because of their biological desired quantitative traits that play key roles in milk production. In this study, three genes directly related to milk production: prolactin (*PRL*), bovine kappa-casein (*K-CN*) and the pituitary-specific transcription factor (*PIT-1*) were analyzed in 144 cows. The aim of this study was to identify polymorphisms in the Holstein-Friesian cattle breed in Palestine in relation to the genetic markers and allelic variants of the three genes. Collection of samples depended on an experimental design that was completely randomized (CRD) and blood samples were collected from different cities across the West Bank, Palestine. The genotypes were determined through the polymerase chain reaction-restriction fragments length polymorphism (PCR-RFLP) technique. The amplified fragments of *PRL* (294-bp), *K-CN* (530-bp) and *PIT-1* (451-bp) were digested with *RsaI*, *HindIII* and *HinfI*, respectively. Statistical analysis found that the prolactin allelic substitution (AG, GG) played a role in milk production with a p-value of 0.00643 and α (0.001), the AG allele of *PRL* being more favorable for milk production as compared to the GG allele. Genetic variants of the bovine *K-CN* gene played a role in milk production with a p-value of 0.04071 and α (0.01*), the AA allele possessing more positive effect than the BB and AB alleles. Similarly, the allelic substitution of the *PIT-1* gene affected milk production with a p-value of 2.274e-05 and α (0***), the AA allele exercising a more positive effect followed by the AB and BB alleles, respectively. Among the three studied breeds (Friesian, hybrid and local), results show that the Friesian breed possesses higher overall milk production in Palestine as compared to the other two breeds.**

Key words: Prolactin (*PRL*), bovine kappa-casein (*K-CN*), pituitary-specific transcription factor (*PIT-1*), polymerase chain reaction-restriction fragments length polymorphism (PCR-RFLP).

INTRODUCTION

Molecular genetic markers are widely used for the characterization of milk production traits in dairy cattle. They are also used for the detection of genetically inherited diseases and for the determination of the evolution of the desired breeds; thus they can be utilized to improve livestock populations (Kolbehdari et al., 2009). The quantitative trait loci (QTL) analysis in particular is quite helpful in bridging the gap between genes and the

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phenotypic traits that result from them. QTL analysis links two types of information, the phenotypic data (trait measurements) and genotypic data (usually molecular markers) in an attempt to explain the genetic basis of variation in complex traits (Kloosterman et al., 2010). This allows researchers to link certain traits with specific regions of chromosome to identify the number, action, interaction and precise location of these regions.

As a result of the recent advances achieved in genomics and molecular biology techniques and the completion of bovine genome sequence, whole cattle genomes can be screened for QTL using molecular maps to locate traits that can affect, for example, milk production (Kolbehdari et al., 2009). This screening has proven critical for the identification of important traits that can provide linkages of phenotypic data with the genetic polymorphism of three genes associated with milk production in cattle (*PRL, K-CN* and *PIT-1*) (Kloosterman et al., 2010).

Among several hormones that regulate lactation and reproduction, prolactin (*PRL*) is a pleiotropic polypeptide hormone that is synthesized in and secreted from the lactotrophic cells of the anterior pituitary gland in bovines and in other vertebrates (Freeman et al., 2000). This hormone family includes placental lactogen and growth hormone. Due to its important role in determining milk yield and quality, *PRL* is considered an excellent trait locus. Quantitative characteristics and single nucleotide polymorphisms (SNPs) occurring within the prolactin gene have been suggested to influence the chemical composition of milk or at least be an effective DNA marker of a sub region of the dairy cattle genome (He et al., 2006; Kolbehdari et al., 2009; Alfonso et al., 2012). This makes prolactin a favorite marker and an instrumental genetic tool in research targeting enhancement and development of dairy cattle with high milk production qualities (Alfonso et al., 2012). *PRL* gene was identified and mapped to chromosome 23 in bovine and the whole sequence of this chromosome is available at NCBI (AC_000180.1). The *Bos taurus* prolactin precursor (*PRL*) gene complete sequence is 9388 bp long (GenBank, Accession No: AF426315) and is composed of five exons and four introns (Brym et al., 2005). Mature prolactin encodes 199 amino acids (Mehmannavaz et al., 2009).

Several studies have screened the genetic polymorphism of the bovine prolactin gene and reported more than 20 SNPs within *PRL* gene sequence (He et al., 2006; Halabian et al., 2008; Mehmannavaz et al., 2009). Although most of the identified SNPs were either silent mutations and/or located within introns, one important SNP affects exon four and can be recognized by *Rsa1* endonuclease digestion due to polymorphic transition of G into A at position 8398 (Brym et al., 2005). This SNP has become a popular genetic marker tool commonly used for genetic characterization and identification of possible linkage associations between *PRL* gene and milk performance traits (Chung et al., 1996; Dybus, 2002; He et al., 2006; Othman et al., 2011).

Casein is a milk protein secreted by mammary gland cells. It constitutes about 78-82% of bovine milk protein, and is divided into four main groups: αS1casien, αS2 casein, β-casein and κ-casein (Azevedo et al., 2008). The beta-lactoglobulin (*B-lg*) and kappa-casein (*K-CN*) are considered two of the most important milk proteins due to their crucial role in milk quality, coagulation process in cheese, butter and the formation, stabilization, and aggregation of the casein micelles (Remus-Alexandru et al., 2000). Nevertheless, K-CN possesses specific quality roles in milk more than B-lg and constitutes approximately 12% of the total casein. The genetic variations of *K-CN* gene allows it to play important roles as a protein milk marker (El-Rafey et al., 2008).

The bovine *K-CN* is located on chromosome 6q31 with an overall length of approximately 13 kb. The *K-CN* gene contains five exons and four introns with most of the coding sequence of the mature K-CN protein located in the fourth exon (Ferretti et al., 1990). The point mutation in exon four of kappa-casein (*CSN3*) gene results in two allelic variations: A and B (Ferrettiet al., 1990). Although nine variants have been described in *K-CN* gene: A, B, C, E, F, G, H, I and A1, the frequent alleles are the A and B variants (Prinzenberg et al., 1999). The A and B variants occur in amino acids located relatively close to several glycosylation sites such as amino acids in position 136 and 148 aa of primary structure. In this variation, threonine is replaced by isoleucine in position 136 aa, whereas aspartic acid is replaced by alanine in position 148 aa for A and B, respectively (Otaviano et al., 2005; Azevedo et al., 2008).

Pituitary specific transcription factor (*PIT-1*) gene has been identified as a regulator of the expression of the growth hormone (*GH*) and prolactin (*PRL*) gene in the anterior pituitary (Herr et al., 1988; Rosenfeld, 1991). The *PIT-1* gene is known by different names; pituitary-specific positive transcription factor 1, growth hormone factor 1, pituitary growth factor, POU domain class 1 and transcription factor 1 (Selvaggi and Dario, 2011). The *PIT-1* (official nomenclature- POU1F1) is a member of the POU-family and it is named so because the first 3 members identified were *PIT-1* and *OCT-1* (MIM 164175) in mammals and Unc-86 of *Caenorhabditis elegans* (Herr et al., 1988), which were transcription factors that regulate mammalian development (Herr et al., 1988; Tang et al., 2012). In mammals, POU1F1 mutations have been found to be associated with mice Snell dwarf and Jackson dwarf mutants and also result in human dwarfism (Pfaffle et al., 1992). The POU1F1 gene was studied in many domestic animals including cattle and is located on chromosome bands 1q21-q22 (Woollard et al., 1994), and in porcine was marked to 13q46.

The genetic variations of *POU1F1* gene in cattle and

porcine are considered associated with important economic traits including production performance (Renaville et al., 1997; Stančeková et al., 1999; Zhao et al., 2004). This is supported by the QTL analysis which revealed that the region surrounding POU1F1 on chromosome 1q21-q22 affects cattle production (Woollard et al., 1994), a direct indication of POU1F1 gene potential consideration in growth trait analysis (Pan et al., 2008; Selvaggi and Dario, 2011). The *PIT-1*cDNA was sequenced and made available to the public in 1988 (Woollard et al., 1994). Studies on *PIT-1* cDNA sequences sub-localized the gene to the centromeric region of the bovine chromosome1 located midway between TGLA57 and RM95 (AC_000158.1, 35008949..35024718, complement) (Dybus, 2002).

The PIT-1 protein is approximately 33 kilodalton with two functional domains: the POU-specific and POU-hemeo. Both are needed for high DNA binding affinity to GH and *PRL* gene promoters (Herr et al., 1988; Rosenfeld, 1991). The PIT-1 is activated in part by the N-terminal trans-activation domain, which is rich in hydroxylated amino acid residues (Dybus el al., 2004).

It was reported that the inhibition of PIT-1 synthesis has resulted in decreased GH and PRL expression and the proliferation of somatotropic and lactotropic cell lines (Dybus et al., 2004; Tang et al., 2012). Similar to *PRL* and *K-CN*, several polymorphisms were identified in the cattle *PIT-1* locus (Dybus, 2002). The first polymorphism is on exon 6 characterized by a substitution of an adenine with a guanine (A207G) located in the *HinfI* restriction site. This SNP is used to characterize the A and B alleles, respectively (Renaville et al., 1997). The second polymerphism is located on exon 3 consisting of several polymerphisms identified in *PIT-1* locus: one located in exon 2, two located in intron 3, one in intron 4 and one in intron 5 (Renaville et al., 1997).

Several studies have suggested that the *PIT-1* polymorphisms play a key role in milk yield and, to a lesser extent, in determining the fat percent in dairy cattle (Dybus et al., 2004; Javanmard et al., 2005). The A allele of *PIT-1*, however, was found to be superior for milk and protein yield as compared to fat percentage in dairy cattle (Renaville et al., 1997; Dybus et al., 2004).

Dairy products are essential components in food industries and individual nutrition in Palestine. Cattle provide the majority of milk used in local dairy industries. There are several standard cattle farms in Palestine, mostly in the Hebron area, and there is an increasing demand on milk in Palestine to meet the growing needs of the society for dairy products. The annual milk yield per cow, however, is lower than that of the Israeli cattle and neighboring countries, suggesting that Palestinians are either using less valuable nutritional feed or that milking cows are not genetically favorable for high milk production. The overall objective of this study was to determine the genetic disposition of milking cattle in representative Palestinian farms specifically focusing on three genes directly related to milk production: *PRL*, *K-CN* and *PIT-1*and provide data that will help in improving cattle breeding and farming styles in Palestine.

MATERIALS AND METHODS

Animals

A total of 101 blood samples of healthy Holstein-Friesian breed females were collected from different farms in Jenin, Tubas, Tumon, Nablus and Hebron in the West Bank, Palestine.In addition to Friesian breed, 18 hybrid and 25 local cows were investigated for allele frequencies.Information on milk production and yield was obtained from the databases of the studied farms for each animal tested.

Genomic DNA extraction

DNA was isolated from the buffy coat, the white layer of white blood cells (WBCs) after centrifugation, which is located in the middle layer between the plasma- supernatant and red blood cells (RBCs) pellet. It was done by using the EZ-DNA Isolation Reagent method from Biological Industries (Cat No 20-60050). Blood samples were collected in 5 ml tubes containing EDTA to prevent coagulation and then centrifuged at 5000 rpm for 10 minutes at 4°C to precipitate white blood cells (buffy coat). After the separation of 300 µl of buffy coat in 2 ml Eppendorf tubes, 800 µl 2 X (RBC) lysis buffer were added. The RBC lysis buffer was prepared by adding 7.7 g NH_4CL and 0.1g $KHCO_3$ in 1 L of distilled water. Tubes were mixed by inversion and incubated for 10 min at 37°C (water bath) before centrifugation at 1300 rpm for 30 s for the first wash. The pellet was then resuspended in 800 µl 2 X RBC lysis buffer by vortexing and the pellet was collected by centrifugation at 1300 rpm for 30 s as a second wash. To lyse the white cells, 1 ml EZ-DNA Isolation Reagent was added to the pellet, resuspended before incubation for 5 min at RT. Following incubation, 1 ml of absolute (99.9%) ethanol was added and the mixture mixed gently by inversion. The DNA was collected by centrifugation at 1300 rpm for 30 s and washed twice by 70% ethanol. (cytosine/adenine (C577A), whereas the third was dissolved in 50 µl TEB buffer and the quality of DNA was assessed by agarose gel electrophoresis and the remaining DNA was stored at -20°C.

Primers used for the amplification of the *PRL, K-CN* and *PIT-1* genes

Primers used in the present study (Sigma Aldrich) were according to available cattle gene sequences, which show high degree of nucleotide sequence conservation between the cattle. We used the BLAST N program to ensure the specificity of forward and reverse primers for the three genes (*PRL, K-CN* and *PIT-1*) studied. All primers showed 100% specificity of forward and reverse primers for the three genes (*PRL, K-CN* and *PIT-1*). The information in relation to PCR primers and restriction enzyme analyses used in the present study is shown in Table 1.

PCR amplification of *PRL, K-CN* and *PIT-1* genes

The amplification of *PRL* gene-294 bp, *K-CN*, 530 bp and 451 bp for *PIT-1* was done with a PCR reaction containing 1 X buffer, 25 mM $MgCl_2$, 1 µM dNTPs, 1 unit Taq DNA Polymerase, 0.5 µM primers (forward and reverse) and approximately 50 ng of template

Table 1. Primer sequences and restriction enzymes used in this study.

Gene	Primer sequence	Annealing temperature (°C)	Restriction enzyme used	Reference
PRL	F- CCA AAT CCA CTG AAT TAT GCT T R- ACA GAA ATC ACC TCT CTC ATT CA	58	RsaI	Brym et al. (2005)
K-CN	F -ATA GCC AAA TAT ATC CCA ATT CAG T R- TTT ATT AAT AAG TCC ATG AAT CTT G	57	HindIII	Denicourt et al. (1990)
PIT-1	F -AAA CCA TCA TCT CCC TTC TT R- AAT GTA CAA TGT GCC TTC TGA G	56	HinfI	Renaville et al. (1997)

genomic DNA in a 25 µl reaction. The PCR conditions were as follows: initial denaturation of 2 min at 94°C, followed by 36 cycles of 30 denaturation at 94°C, 30 s annealing at 56°C, 45 s extension at 72°C and a final extension of 10 min at 72°C. The presence of PCR products was confirmed by analysis on 1.5% agarose gel electrophoresis. A 5 µl aliquot from each PCR reaction was loaded on the gel and a 1500 bp ladder was used to determine the fragment size. The gel was visualized using UV fluorescence and photographed by a digital camera. To verify the sequence of amplified fragment for all three genes, the PCR products were amplified, purified and sequenced using the Heredity Lab in Bethlehem University. Results of the three genes: *PRL, K-CN* and *PIT-1* were blasted against available GenBank sequences and checked by BLAST N version 4 program to generate alignments for SNP identification.

Enzyme digestion and detection of genotypes

The PCR amplified 294 bp of *PRL* product was digested by *Rsa1* restriction enzyme in a digestion reaction consisting of 10 µl PCR product, 2 µl 10 x buffer, 2 µl enzyme in a final volume of 32 µl. For the *K-CN* and *PIT-1* genes, the amplified PCR products of 530 and 451-bp were digested by *HindIII* and *HinfI* restriction enzymes, respectively, as described for PRL above. The digestion reactions for the three enzymes were: incubation for 16 h at 37°C in a thermocycler to control temperature. Following incubation, the digestion mixture was loaded on 3% agarose gel and visualized under UV as described above. The sizes of the resulting fragments were measured for each digestion.

Statistical analysis

One way analysis of variance (ANOVA) analysis was conducted to compare the yield of milk with the *PRL*, *K-CN* and *PIT-1* gene frequencies. Information on sample numbers and significance are provided in the figure captions.

RESULTS AND DISCUSSION

There are three cattle breeds, Friesian, local and hybrid that exist in Palestine and are used mainly for milk production and meat. Dairy industries purchase milk from cattle farms and to a lesser extent from individual farmers who grow small numbers of cattle in their farms. The south of Palestine, mainly in Hebron area, is considered the center of dairy industries with more than 17 farms that employ some type of management including a database for their cattle. Cows are milked two to three times per day on most farms; with most farms milking their cows 3 times per day because there is an estimated 10% increase in milk production that can be obtained.

In Palestine, milk is taken either row or processed into a variety of dairy products; the most common in Palestine are the cheese, yoghurt, labaneh, and Jamid, which is a dried, salty processed milk, very common in the West Bank (Ishnaiwer and Al-Razem). There are different preferences for milk in terms of yield, protein and fat contents, but in Palestine, the milk yield was always the major selection criteria favored by dairy cow owners and breeders. Before this study, however, the selection for high yielding dairy cows was based either on the farmer's observation of individual cow yield and body morphology or on the information received from the sellers on the grown dairy cow. During the course of this study, we have not found farms that would employ or take the genotypic variations of the dairy cows into consideration in relation to milk yield. This is likely because of the lack of awareness among cattle growers on the role and importance of these genetic variations in milk production. Most cattle farms in Palestine are owned and managed by the same family members who own the farm and these farms rarely employ full-time veterinarians and animal breeders in their farms.

Tracking the genes in cattle which are known to be associated with milk properties can identify the status of the breed in relation to the milk trait under consideration. Farmers can then manage the breed based on the genetic variations that can be used to select specific criteria relative to demands in the dairy industries. Since most of the traits in relation to milk properties are not controlled by a single gene, this study has considered three different genes related to milk traits, the *PRL, K-CN* and *PIT-1* that are known to be associated with milk yield in dairy cattle. Furthermore, while focusing on one SNP in these genes (*PRL- 294bp, K-CN-530 pb, PIT-1*-451 *bp*), the possible interrelations between these genes have been analyzed.

PCR amplification of milk genes

High quality genomic DNA was used as a template for the amplification of the three milk genes: *PRL, K-CN* and

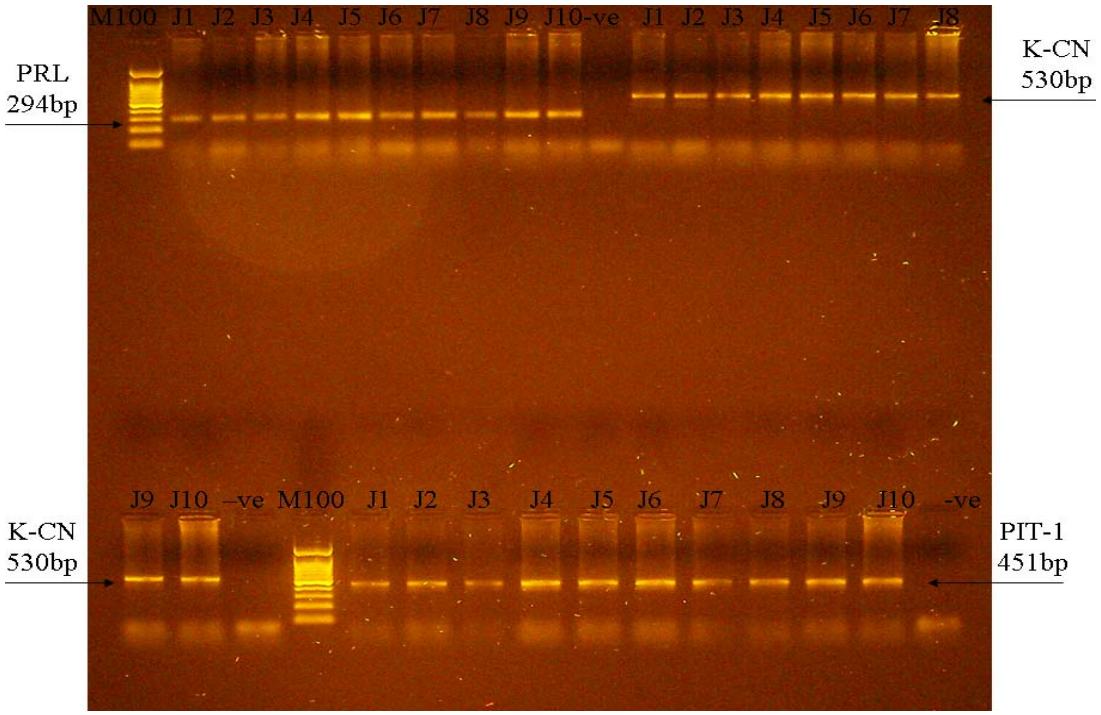

Figure 1. A representative PCR amplification of three milk genes investigated in Palestinian cattle in this study. The PCR products of the three milk genes (*PRL, K -CN, and PIT-1*) were amplified using appropriate primers and genomic DNA template from blood samples collected from cows from Jenin area (J1-J10). Bands as seen on a 1.5% agarose gel stained with EtBr are of the expected sizes for *PRL* -294 bp (top left), *PIT-I* -451 bp (bottom right), and *K-CN* -530 bp (top right and bottom left). A negative control (-ve) lacking genomic DNA template was run for each gene as shown. Lanes labeled with the M100 are the 100 bp DNA ladder.

PIT-1. All three amplicons were of expected correct sizes: 294 bp for *PRL*, 530 bp for *K-CN* and 451bp for *PIT-1* (Figure 1). The amplicons of the three genes (*PRL, K-CN* and *PIT-1*) were presented (Figure 1) and sequence results clearly indicate that the correct target genes were investigated in this study.

Genotypic analysis of *PRL*, *K-CN* and *PIT-1*

The quantity and quality of DNA are quite important for a successful RFLP analysis. Following quantification of DNA on a spectrophotometer, the quality of bands as appeared on agarose gels was fundamental in deciding which DNA to be used for further RFLP analysis. As described above, the three genes (*PRL, K-CN* and *PIT-1*) were amplified using appropriate primers and samples of the PCR products were loaded on 1.5% agarose gel for quality checkup.

Genotyping of PCR products of *PRL*, *K-CN* and *PIT-1* genes was done by using PCR-RFLP method. Below are the results of the genotypic analyses of the three genes from DNA selected from Hebron samples (h1-h31).

PRL gene

After amplification, PCR products were digested with *Rsa1* restriction enzyme. Since the *PRL* amplified region was a small amplicon of only 294 bp, the digested fragments of 162 and 132 bp appeared close to each other on the gel, but it was possible to see the resulted two alleles, GG and AG (Figure 2). Genotypic analysis of the *PRL* gene showed that two genotypes were presented for this gene, the homozygous (GG) genotypes appeared as one undigested band of 294 bp and the heterozygous (AG) genotypes appeared as two digested bands of 163 and 132 bp in addition to the undigested 294 bp G allele (Figure 2).

Polymorphism of prolactin gene was analyzed as a candidate gene responsible for variation and genetic trends in milk yield and composition. The SNP of G into A has become a popular genetic marker tool commonly used for genetic characterization and identification of possible linkage associations between PRL gene and milk performance traits (He et al., 2006). ANOVA analysis revealed significant correlation between the allelic substitution (AG, GG) and milk production with p-value =

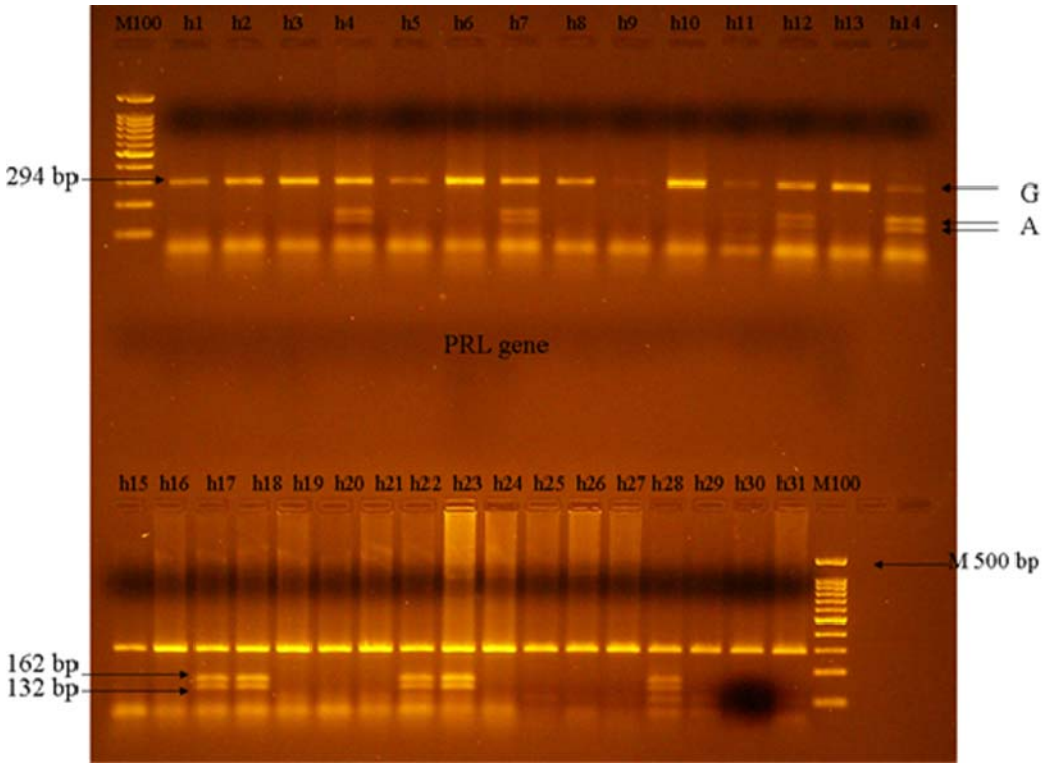

Figure 2. The genotypic analysis of *PRL* gene in Palestinian cattle. PCR products amplified from genomic DNA collected from blood samples extracted from Hebron city cattle (h1- h31) and digested with *Rsa1* before loading on a 3% agarose gel as described above. Alleles revealed by the genotypic analysis were the GG and AG. The homozygous (GG) genotypes appeared as undigested one band of 294 bp, whereas the heterozygous (AG) genotypes appeared as digested two bands (163 - 132 bp) and one undigested G allele. Lanes labeled (h4, h7, h12, h14, h17, h18, h22, h23 and h28) were all AG heterzygous genotype, while the lanes lablled (h1-h3, h5, h6, h8-h11, h13, h15, h16, h19-h21, h24-h27 and h29-h31) were all homozygous GG genotype. Lanes labeled with the M100 are the 100 bp DNA ladder.

0.00643 and α (0.001**). The GG allele was unfavorable for milk production with average mean less that 9000 L per 305 day, whereas the AG allele was shown to be more favorable for milk production with average mean of more than 11000 L (Figure 3).

Since the *PRL* gene is considered a genetic marker for production traits in dairy cattle (Alipanah et al., 2007), the gene has been cloned and characterized in many other animal species (Li et al., 2006) and genetic screening for polymorphisms in bovine prolactin gene identified more than 20 SNPs within the gene sequence (He et al., 2006; Halabian et al., 2008; Mehmannavaz et al., 2009). Most of the identified SNPs were, however, either silent mutations and/or are located within introns. The SNP that was used in this study is the most popular genetic marker tool and is commonly used for genetic characterization and identification of possible linkage associations between the *PRL* gene and milk performance traits (Chung et al., 1996; Dybus, 2002; He et al., 2006). Association of *Rsal/PRL* variants with milk related traits was confirmed in different studies on several cattle breeds such as Jersey cows (Brymet al., 2005) and Russian Red Pied cows (Alipanahet al., 2007). The *PRL* allelic variations were analyzed also in Iranian Holstein bulls (Mehmannavaz et al., 2009). The frequencies reported for A and G alleles were 0.069 and 0.931, respectively. The allelic substitution effect was significant for milk and protein yield ($p < 0.05$) where the G allele was unfavorable for milk and protein yield (Mehmannavaz et al., 2009). Ours results show that all 101 tested Holstein-Friesian for allele frequencies of A and G were 0.28 and 0.71, respectively (Table 2), thus different from frequencies by Brym et al. (2005) who reported 0.11 and 0.88 for A and G, respectively for black-white cows and 0.70 and 0.29 for A and G, respectively, in Jersey cows. Black and White cows with genotype AG showed the highest milk yield, while cows with genotype GG showed the highest fat content. The high frequencies of G allele were reported in other cattle breed including the Brown Swiss (0.61) and Holstein breed (0.95) (Chrenek et al.,

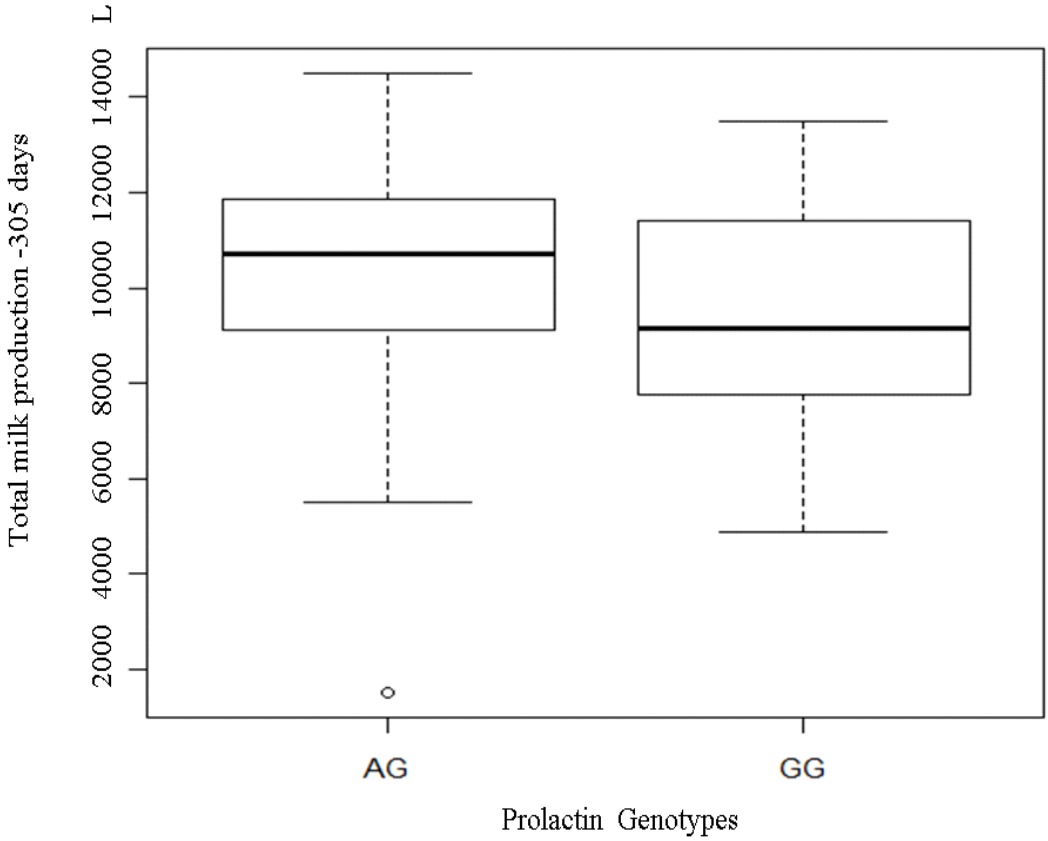

Figure 3. Analysis of *PRL* allelic substitution effect on milk production. As shown, the AG allele was more favorable for milk production with an average mean of more than 11000 L as compared to the less favorable GG allele with an average mean of less than 9000 L milk produced per 305 days. The *PRL* allelic substitution (AG, GG) effect is significant for milk production with p-value = 0.00643 and α = 0.001 ** as analyzed by ANOVA on 101 blood samples.

Table 2. Allele frequencies and genotypes for the three population cattle breeds studied in 144 dairy cows. (101 Friesian and the remaining 43 from local and hybrid cows). The observed and expected heterozygosities are also included.

Loci	Population	N	Allele Frequencies		Genotypes (observed number)			Expected heterozygosity	Observed heterozygosity
			G	A	GG	AG			
PRL	Friesian	101	0.7128	0.2871	43	58		0.4092	0.4257
	Hybrid	18	0.9444	0.0556	16	2		0.1050	0.8888
	Local	25	0.8200	0.1800	16	9		0.2952	0.6400
			A	B	AA	AB	BB		
K-CN	Friesian	101	0.8019	0.1980	66	30	5	0.3175	0.7029
	Hybrid	18	0.7500	0.2500	10	7	1	0.3750	0.6111
	Local	25	0.8400	0.1600	19	4	2	0.2688	0.8400
			A	B	AA	AB	BB		
PIT-1	Friesian	101	0.6831	0.3168	52	34	15	0.4328	0.6633
	Hybrid	18	0.3333	0.6666	3	6	9	0.4439	0.6666
	Local	25	0.2200	0.7800	1	9	15	0.3432	0.6400

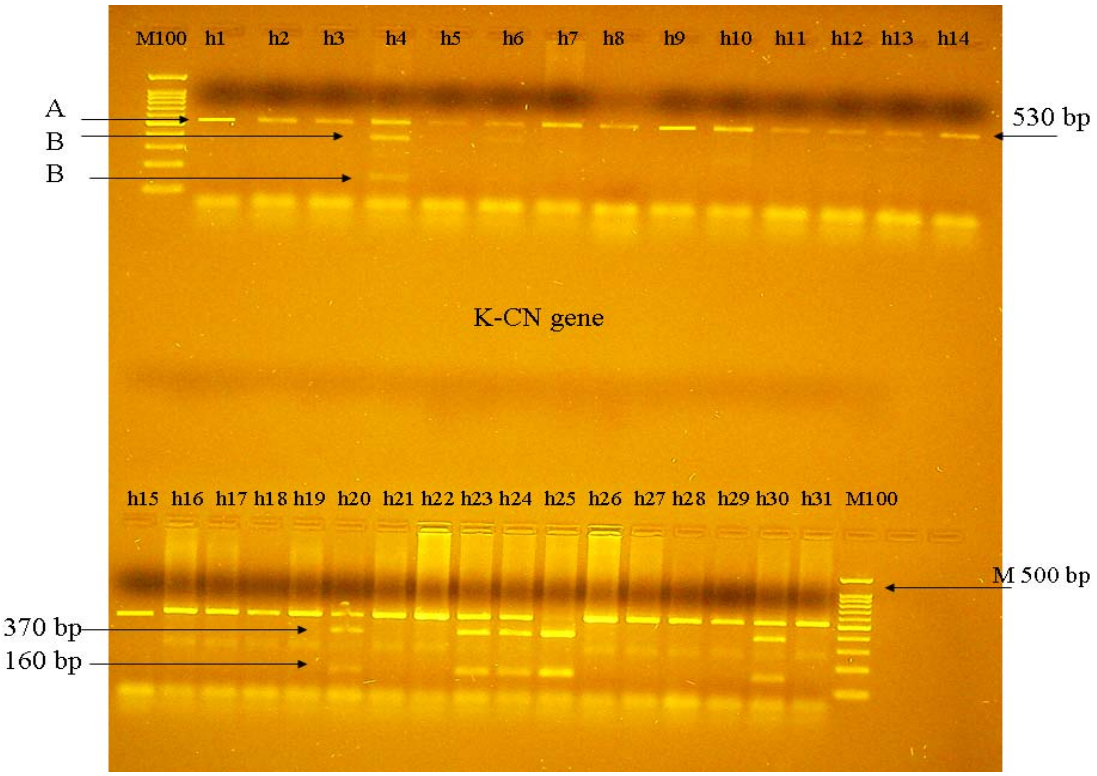

Figure 4. The genotypic analysis of *K-CN* gene in Palestinian cattle. PCR products amplified from genomic DNA collected from blood samples extracted from Hebron city cattle (h1- h31) and digested with *HindIII* before loading on a 3% agarose gel as described above. Alleles revealed by the genotypic analysis were the AA, AB and the BB. The homozygous (AA) genotype appeared as undigested one band of 530 bp, whereas the heterozygous (AB) genotype appeared as one undigested 530 bp fragment and two digested bands of 370 and 160 bp.The BB restriction bands were 370 and 160 bp. Lanes labeled (h1-h3, h5, h7-h19, h21, h22, h26-h29 and h31) are AA, whereas h4, h6, h20, h23, h24, h30 are AB genotype. The BB genotype appeared in lane (h25). Lanes labeled with the M100 are the 100 bp DNA ladder.

1998). The differences in genotypes are likely due to long-term artificial inseminations and selection towards high milk production and quality.

K-CN gene

The *K-CN* gene was amplified using PCR amplification procedures. PCR products were digested with *HindIII* restriction enzyme. The *K-CN* amplified region showed a 530 bp amplicons on gel (Figure 1). Digestion analysis of the *K-CN* gene revealed three genotypes (Figure 4): the homozygous AA as undigested band of 530 bp, the heterozygous AB genotype as three bands one of 530 bp and two digested bands of 370 and 160 bp for the B allele. The third was the homozygous BB genotype consisted of two bands, one undigested 530 bp and one digested shorter band of 160 bp (Figure 4).

Polymorphism of *K-CN* gene was analyzed using ANOVA. *K-CN* genetic variations (for example, AA, AB, BB alleles) strongly associated with differences in milk composition, processing properties, and thus affecting dairy products.

The SNP of *K-CN* has become a popular genetic marker tool commonly used for genetic charac-terization and identification of possible associations between the *K-CN* gene and milk performance traits. ANOVA analysis revealed that the allelic substitution (AA, AB, BB) effect was significant for milk production with p-value = 0.04071 and α (0.01 *). The AA and BB alleles were favorable for milk production, whereas the AB allele was less favorable for milk production (Figure 5).

The *K-CN* gene possesses specific quality roles in milk more than β-Lg and its protein product constitutes approximately 12% of the casein in milk. It can play an important role in marker assisted selection of milk trait (Azevedoet al., 2008). The *K-CN* locus has been shown in different genome variations strongly associated with differences seen in milk composition and processing properties that affect dairy products (Riaz et al., 2008).

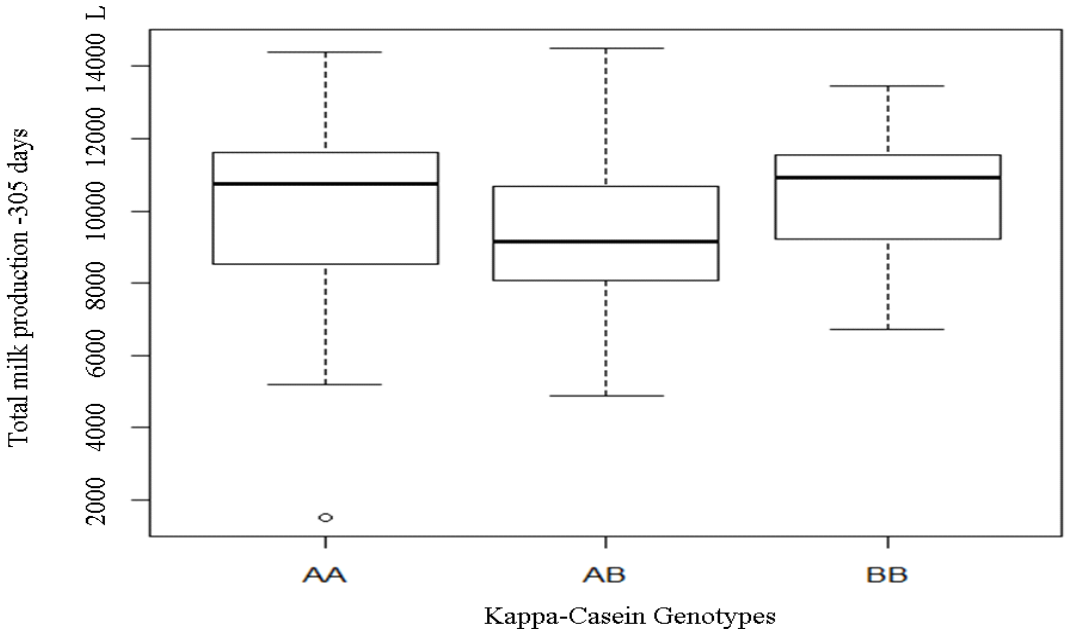

Figure 5. Analysis of *K-CN* allelic substitution effect on milk production. As shown, the AA and BB alleles were more favorable for milk production with an average mean of more than 11000 L as compared to the less favorable AB allele with an average mean of less than 9000 L milk produced per 305 days. The *K-CN* allelic substitutions (AA, AB and BB) effect is significant on milk production with p-value = 0.04071 and α = 0.01 * as analyzed by ANOVA on 101 blood samples.

Genetic variants of bovine *K-CN* gene are associated with protein content of milk and have influenced rennet clotting time, firmness and cheese yield of milk with a superiority of milk from cows with *K-CN/BB* as compared to *K-CN/AA* genotype as shown in previous studies (Marziali and Ng-Kwai-Hang, 1986). In the present study, both AA and BB alleles had approximately similar mean average of milk production that was higher than the AB allele (Figure 5). The allele frequencies were 0.80 and 0.19 for A and B, respectively. (Table 2) The association of *HindIII-K-CN* variants with milk related traits was confirmed in other studies carried out on several breeds (Denicourt et al., 1990). The allelic variants of the *K-CN* gene in Sahiwal and Tharparkar cattle breeds were analyzed (Rachagani and Gupta, 2008). The *K-CN/BB* genotype had more influence on the milk, fat, and protein yield in the Sahiwal cattle. According to Marziali and Ng-Kwai-Hang (1986), cheese production can be increased by 10% if milk is from a cow of the *K-CN/BB* genotype as compared to *K-CN/AA* genotype. Therefore, it has been proposed to increase the frequency of *K-CN/BB* genotype in breeding programs preferring sires with the *K-CN/BB* genotype. The effect of *K-CN* polymorphism on milk performance traits was also studied in Holstein-Friesian heifer cows (Beata et al., 2008). However, in contrast to studies that suggested the association between *K-CN/BB* genotype and high milk yield (Rachagani and Gupta, 2008), the authors reported that the *K-CN/AA* genotype was characterized by the highest milk, fat and protein yield, whereas *K-CN/BB* genotype showed the lowest fat and protein contents in their milk (Beata et al., 2008). This is in agreement with the current study and that of Curi et al. (2005), where the association between *K-CN/AA* genotype and high milk production was observed. It also points to the involvement of other factors in milk production besides the K-CN.

PIT-1 gene

The *PIT-1* gene was amplified, PCR products were digested with *HinfI* restriction enzyme. The *PIT-1* amplified region showed a 451 bp amplicon on gel (Figure 1). Digestion analysis of the *PIT-1* gene revealed three genotypes (Figure 6): the homozygous AA as undigested band of 451 bp, the heterozygous AB genotype as three bands; one undigested of 451 bp and two digested bands that were close to each other with sizes of 207 and 244 bp. The third was the homozygous BB genotype consisting of two digested, but shorter bands of 207 and 244 bp (Figure 6).

The *PIT-1* genetic variations (for example, AA, AB, BB genotypes) are strongly associated with differences in milk yield and animal growth. The SNP of *PIT-1* has

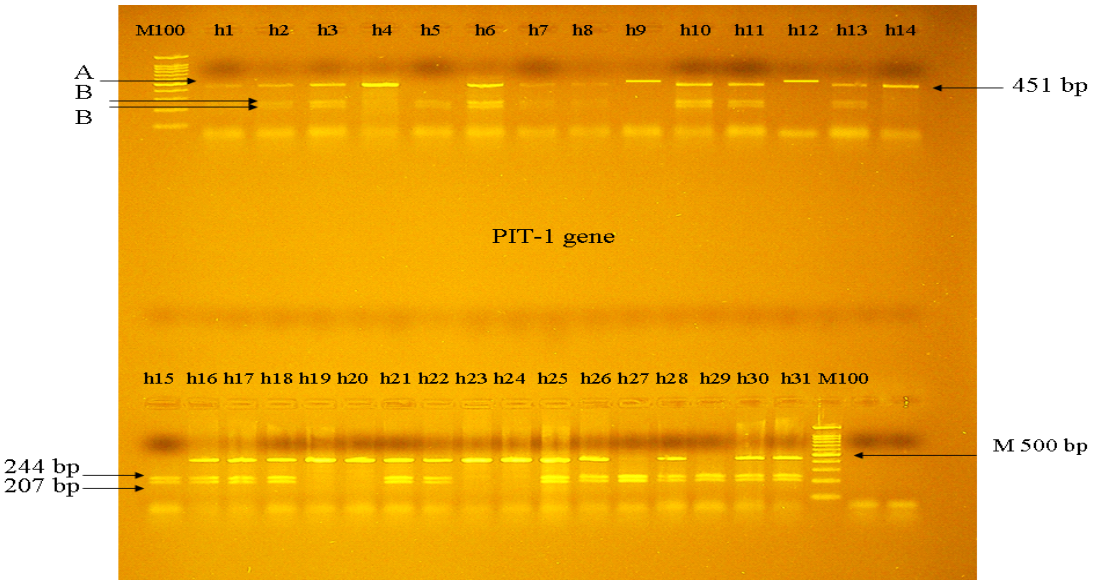

Figure 6. The genotypic analysis of *PIT-1* gene in Palestinian cattle. PCR products amplified from genomic DNA collected from blood samples extracted from Hebron city cattle (h1- h31) and digested with *HinfI* before loading on a 3% agarose gel as described above. Alleles revealed by the genotypic analysis were the AA, AB and the BB. The homozygous (AA) genotypes appeared as one undigested band of 451 bp, whereas the heterozygous (AB) genotypes appeared as one undigested 451 bp fragment and two digested bands of 207 and 244 bp. The BB restriction bands appeared as 244 and 207 bp fragments. Lanes labeled (h1, h9, h12, h14, h19, h20, h23, h24) are the homozygous AA, whereas the heterzygous AB are in lanes (h2, h3, h6-h8, h10, h11, h13, h16, h18, h21, h22, h25, h26, h28, h30 and h31) and for the BB genotype, lanes (h5, h15, h27 and h29). Lanes labeled with the M100 are the 100 bp DNA ladder.

become a popular genetic marker tool commonly used for genetic characterization and identification of possible linkage associations between *PIT-1* gene and milk performance traits. ANOVA analysis revealed that the allelic substitution (AA, AB, BB) effect was significant for milk production with p-value = 0.274e-05 and α = 0 ***. The AA was the most favorable allele for milk production with an average milk production of more than 11000 L as compared to the intermediate AB (less than 9000 L) and the lowest favorable BB alleles with an average milk production of less than 8000 L per 305 days (Figure 7).

In cattle, *PIT-1* was found in several studies to be associated with body weight and average daily gains (Renaville et al., 1997; Carrijo et al., 2008) and milk production traits (Renaville et al., 1997; De Mattos et al., 2004; Xue et al., 2006). Other studies, however, failed to verify the association between *PIT-1* and production traits (Di Stasio et al., 2002; Dybus et al., 2004; Zhao et al., 2004; Selvaggi and Dario, 2011).

There are several polymorphic cattle *PIT-1* loci identified. In Holstein breed, it was shown that A allele, characterized in site from exon 6 of *PIT-1* gene, has significant positive effect on production traits in cattle (Carsai et al., 2012). These polymorphisms have been shown to play a key role in milk yield and, to a lesser extent, in determining the fat percentage in dairy cattle (Dybus, 2002; Javanmard et al., 2005). The A allele of *PIT-1* was also found to be superior for milk and protein yield, but inferior for fat percentage in dairy cattle (Renaville et al., 1997; Dybus, 2002). In this study, the *PIT-1* gene polymorphism was shown to play a significant role in milk production, with the *PIT-1*/ AA genotype being more important for milk production than the *PIT-1*/ AB and *PIT-1*/ BB genotype respectively (Figure 7).

The polymorphism within bovine *PIT-1* gene effect on production traits was also reported in several studies (Woollard et al., 1994; Renaville et al., 1997) where the A allele seemed to be linked to higher milk yield and more protein yield but lower fat percentage. Furthermore, Hori-Oshima and Barreras-Serrano (2003) studied the *PIT-1* gene polymorphism in Baja California Holstein cattle and found that the *PIT-1* /AA genotype possessed a significant effect on milk yield (Hori-Oshima and Barreras-Serrano, 2003) similar to what was reported by Renaville et al. (1997) and Viorica et al. (2007), where the A allele was found to be superior for milk and protein yields and inferior for fat percentage in Romanian Simmental cattle (Renaville et al., 1997; Viorica et al., 2007). Allele frequencies in the present study for A and B were 0.68 and 0.31, respectively (Table 2). In studies on Canadian Holstein bulls, the frequency of B allele was found to be 0.79 (Sabour et al., 1996) and 0.812 in

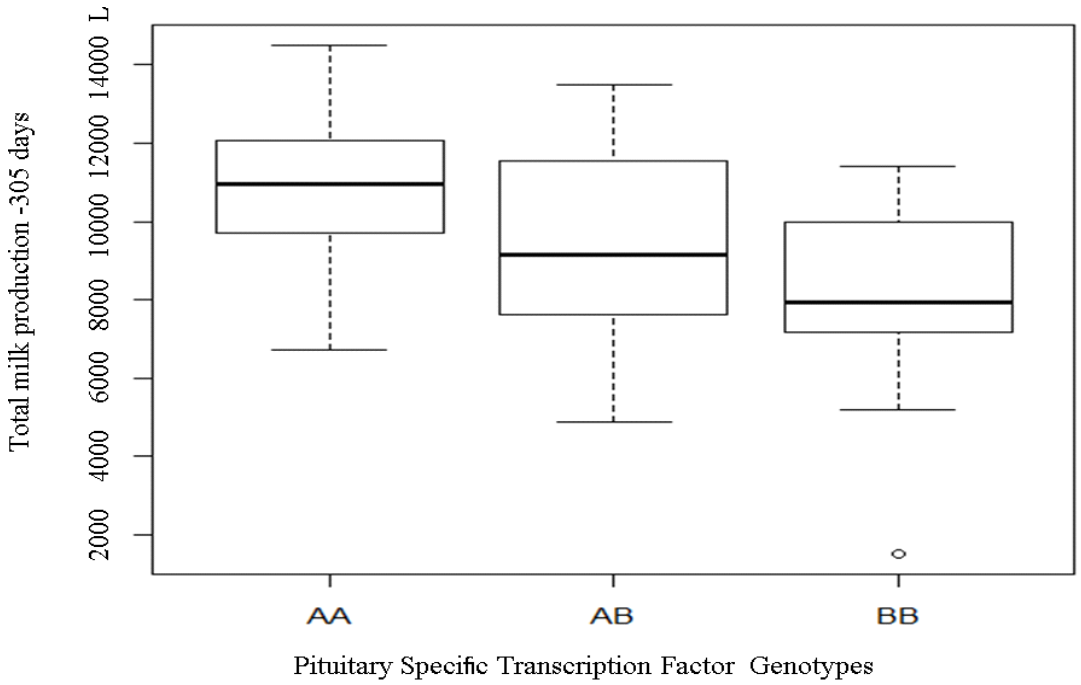

Figure 7. Analysis of *PIT-1* allelic substitution effect on milk production. As shown, the AA allele was the most favorable for milk production with an average mean of more than 11000 L per 305 days compared to the intermediate AB allele with an average mean milk production of less than 9000 L and the least favorable BB allele of an average mean of milk production of less than 8000 L per 305 days. The *PIT-1* allelic substitutions (AA, AB and BB) effect is significant on milk production with p-value = 2.274e-05 and α = 0.0 *** as analyzed by ANOVA on 101 Friesian blood samples.

Italian Holstein Friesian bulls (Renaville et al., 1997). This is slightly higher than the B allele frequencies reported in Polish Black and White cattle, which were very similar in three studies, 0.75 (Klauzin'ska et al., 2000), 0.74 (Oprzadek et al., 2003) and 0.757 (Dybus et al., 2004).

Allele frequencies, heterozygosities and Hardy-Weinberg equilibrium

Allele frequency is a measure of the relative frequency of an allele of a genetic locus in a selected population and can provide us with information on the genetic diversity of the Palestinian dairy cattle. In the present study, the existence of two alleles for the three milk genes was verified in Palestinian cattle, with two alleles for the *PRL* (G and A), two for *K-CN* (A and B), and two for *PIT-1*(A and B). The frequency for each allele was calculated for the three studied populations (Friesian, hybrid and the local baladi cattle) (Table 2).

For the *PRL* gene, two genotypes (GG, AA) were identified and the highest frequency was 0.94 for G allele detected in the hybrid breeds (Table 2). On the other hand, when Friesian breeds were compared with the other two breeds, the highest frequency was found in the G allele of the local breeds (0.8200) and lowest in the Friesian breeds (0.7128). The Friesian breeds possessed the highest frequency (0.2871) for the A allele and the highest expected heterozygosity (0.4092) when compared with local and hybrid breeds for the A allele (0.1800), (0.0556) and expected heterozygosity of 0.2952 and 0.1050, respectively.

For the *K-CN* gene, the frequency of A allele was higher than the B allele in all of the breeds studied (local, Friesian and hybrid- 0.8400, 0.8019, 0.7500, respectively). The hybrid breed possessed the highest expected heterozygosity number of (0.3750) when compared with the Friesian (0.3175) and with the Local breeds (0.2688).

For the *PIT-1* gene, the A allele was higher than the B allele in the three breeds. Friesian breed possessed the highest number in allele frequency (0.6831) as compared to the other two populations, the hybrid (0.3333) and the Local (0.2200) breeds.

Friesian breeds, however, possessed lower expected heterozygosity (0.4328**)** when compared with the hybrid breeds (0.4439), but the highest expected heterozygosity (0.3432) when compared with the Local breeds. Information obtained from the allele frequencies of the three genes in Friesian, hybrid and local cattle can be used as a tool to select the sperms of bulls to achieve im-

provements and enhance the selection of high milk yield dairy cows. As shown in the results, the Friesian breed represents the highest yielding cattle and is the primary selected breed in dairy farms.

In conclusion, advances in molecular genetics have allowed the use of specific DNA markers associated with various productivity traits in promoting efficient selection and breeding strategies of farm animals. For example, many candidate genes like (*PRL*, *K-CN* and *PIT-1*) have been identified and selected for analysis based on a known relationship with productivity traits. According to the results obtained in this study, these genes are in fact good candidates for consideration in programs of marker assisted selection applied for the improvement of cattle milk production in Palestine. Allelic variations for the three genes play key roles in milk production and quality, likely with the allele A considered to be the best indicator for the milk production in the Holstein-Friesian breed.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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