

# Journal of Cell and Animal Biology

Volume 8 Number 5, May 2014

ISSN 1996-0867



*Academic  
Journals*

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

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## Full Length Research Paper

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

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Received 3 March, 2014; Accepted 24 March, 2014

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  $\alpha$  (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  $\alpha$  (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  $\alpha$  (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 *RsaI* 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:  $\alpha$ S1casein,  $\alpha$ S2 casein,  $\beta$ -casein and  $\kappa$ -casein (Azevedo et al., 2008). The beta-lactoglobulin (*B-Ig*) 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-Ig* 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 (Ferretti et 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; Stančková 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 chromosome 1 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 et 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 *Hinfl* restriction site. This SNP is used to characterize the A and B alleles, respectively (Renaville et al., 1997). The second polymorphism is located on exon 3 consisting of several polymorphisms 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 repre-

sentative 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<sub>4</sub>CL and 0.1g KHCO<sub>3</sub> 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<sub>2</sub>, 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
<i>PRL</i>	F- CCA AAT CCA CTG AAT TAT GCT T R- ACA GAA ATC ACC TCT CTC ATT CA	58	Rsal	Brym et al. (2005)
<i>K-CN</i>	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)
<i>PIT-1</i>	F -AAA CCA TCA TCT CCC TTC TT R- AAT GTA CAA TGT GCC TTC TGA G	56	Hinfl	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 s 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 *RsaI* 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 *Hinfl* 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

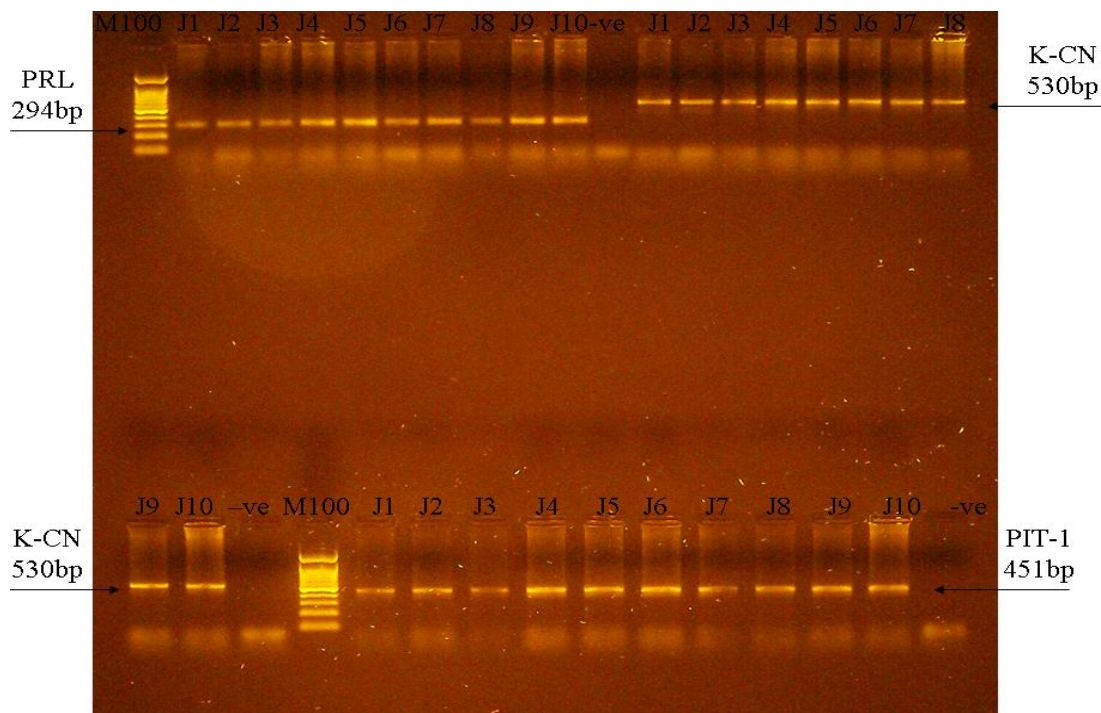
day on most farms; with most farms milking their cows 3 times per day because there is an estimated 10% increase in milk production that can be obtained.

In Palestine, milk is taken either raw 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-1* -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.

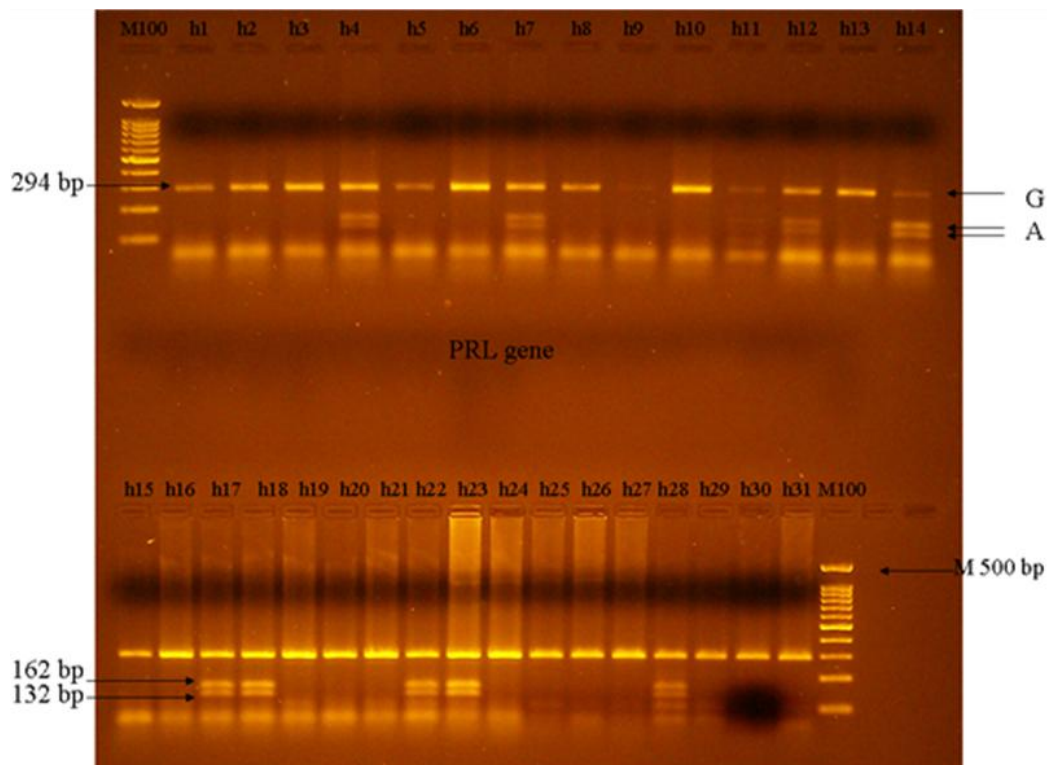
Genotyping of PCR products of *PRL*, *K-CN* and *PIT-1* genes was done by using PCR-RFLP method. Below are the results of the genotypic analyses of the three genes from DNA selected from Hebron samples (h1-h31).

### *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 =



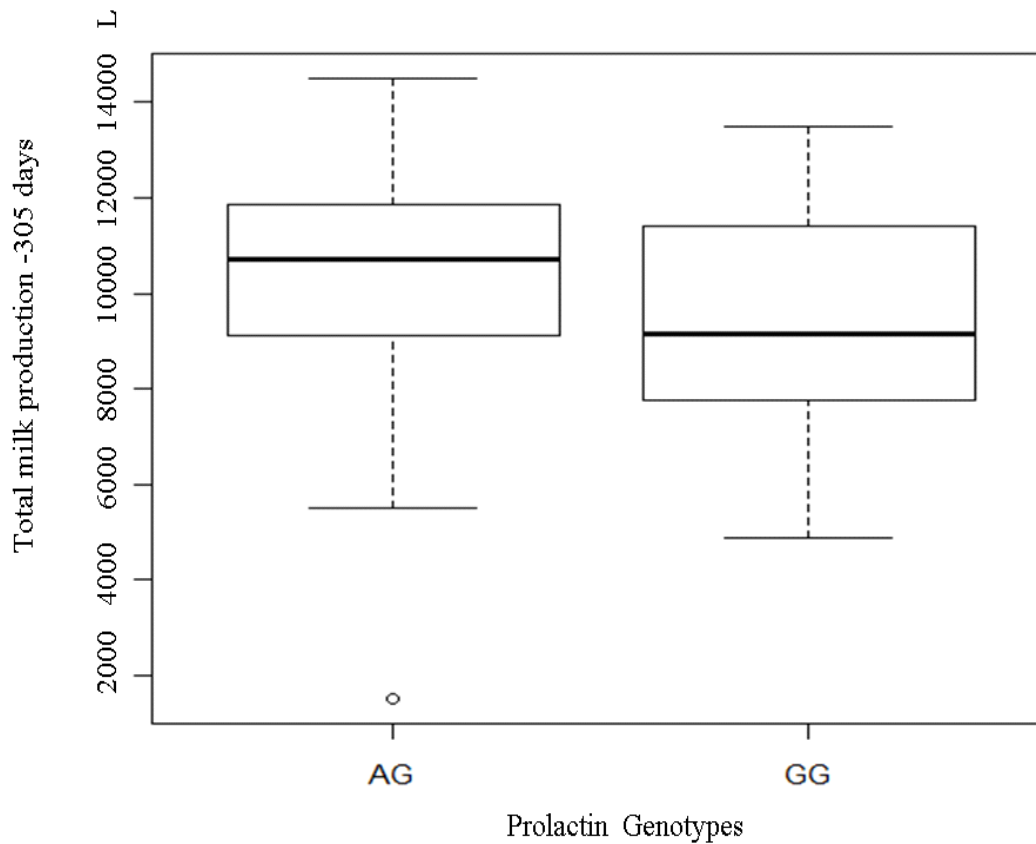


**Figure 2.** The genotypic analysis of *PRL* gene in Palestinian cattle. PCR products amplified from genomic DNA collected from blood samples extracted from Hebron city cattle (h1- h31) and digested with *Rsa1* before loading on a 3% agarose gel as described above. Alleles revealed by the genotypic 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 heterozygous genotype, while the lanes labelled (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  $\alpha$  (0.001\*\*). The GG allele was unfavorable for milk production with average mean less than 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; Mehmannaavaz 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 *RsaI/PRL* variants with milk related traits

was confirmed in different studies on several cattle breeds such as Jersey cows (Brymet et al., 2005) and Russian Red Pied cows (Alipanah et al., 2007). The *PRL* allelic variations were analyzed also in Iranian Holstein bulls (Mehmannaavaz 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 (Mehmannaavaz 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 Brymet 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.,

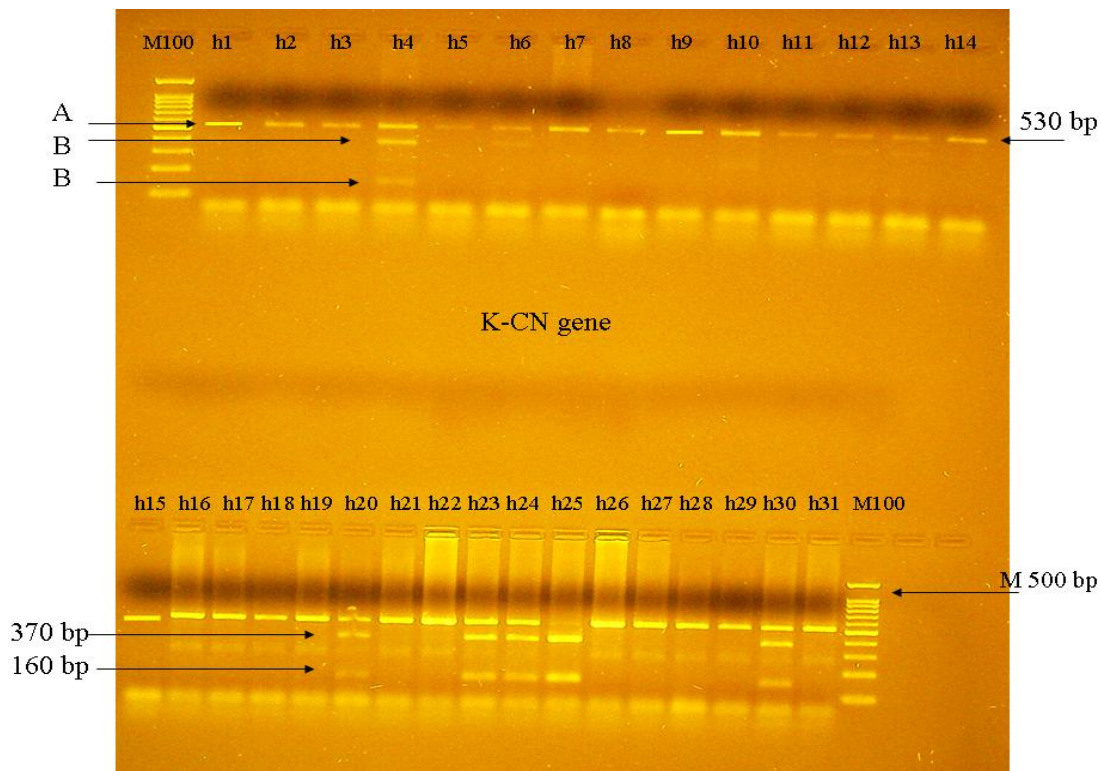


**Figure 3.** Analysis of *PRL* allelic substitution effect on milk production. As shown, the AG allele was more favorable for milk production with an average mean of more than 11000 L as compared to the less favorable GG allele with an average mean of less than 9000 L milk produced per 305 days. The *PRL* allelic substitution (AG, GG) effect is significant for milk production with p-value = 0.00643 and  $\alpha = 0.001$  \*\* 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	BB		
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
K-CN			A	B	AA	AB	BB		
	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	
PIT-1			A	B	AA	AB	BB		
	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**

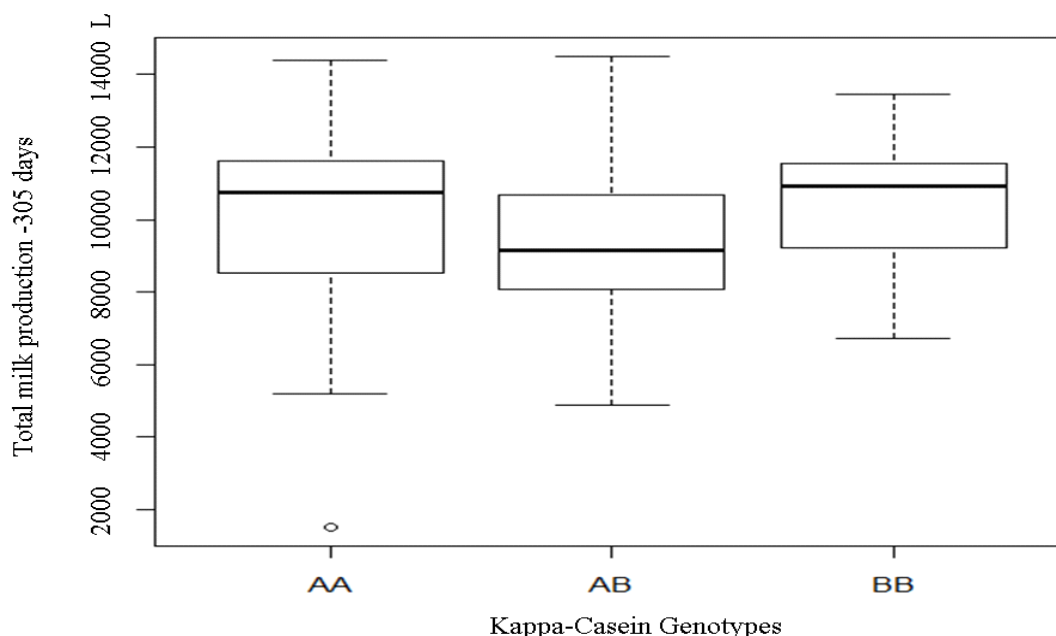
The *K-CN* gene was amplified using PCR amplification procedures. PCR products were digested with *HindIII* restriction enzyme. The *K-CN* amplified region showed a 530 bp amplicons on gel (Figure 1). Digestion analysis of the *K-CN* gene revealed three genotypes (Figure 4): the homozygous AA as undigested 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 characterization 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  $\alpha$  (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  $\beta$ -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 (Azevedo et 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  $\alpha = 0.01$  \* as analyzed by ANOVA on 101 blood samples.

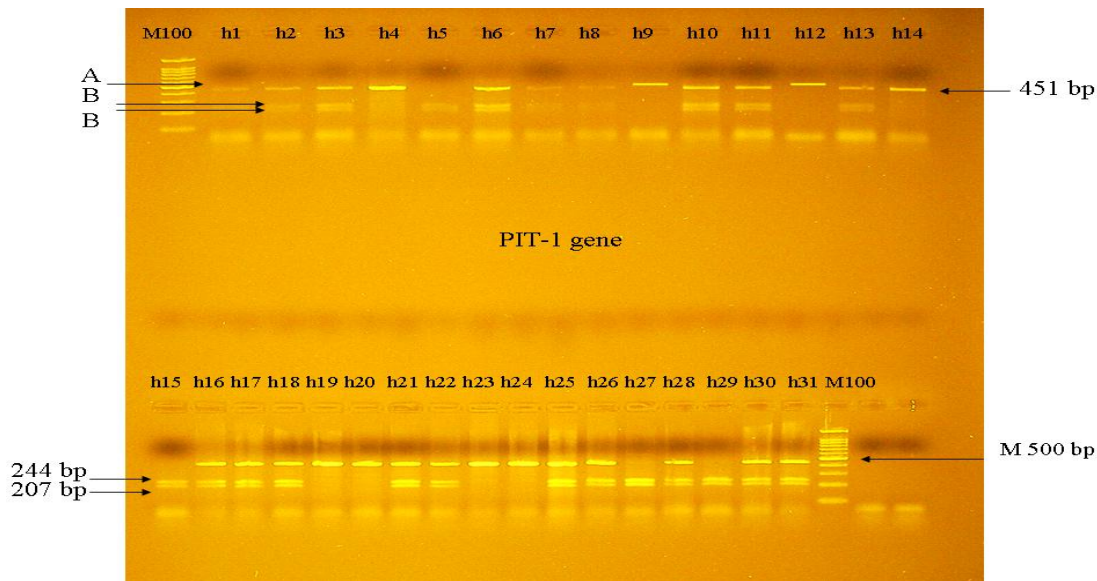
Genetic variants of bovine *K-CN* gene are associated with protein content of milk and have influenced rennet clotting time, firmness and cheese yield of milk with a superiority of milk from cows with *K-CN/BB* as compared to *K-CN/AA* genotype as shown in previous studies (Marziali and Ng-Kwai-Hang, 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 heterozygous 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.

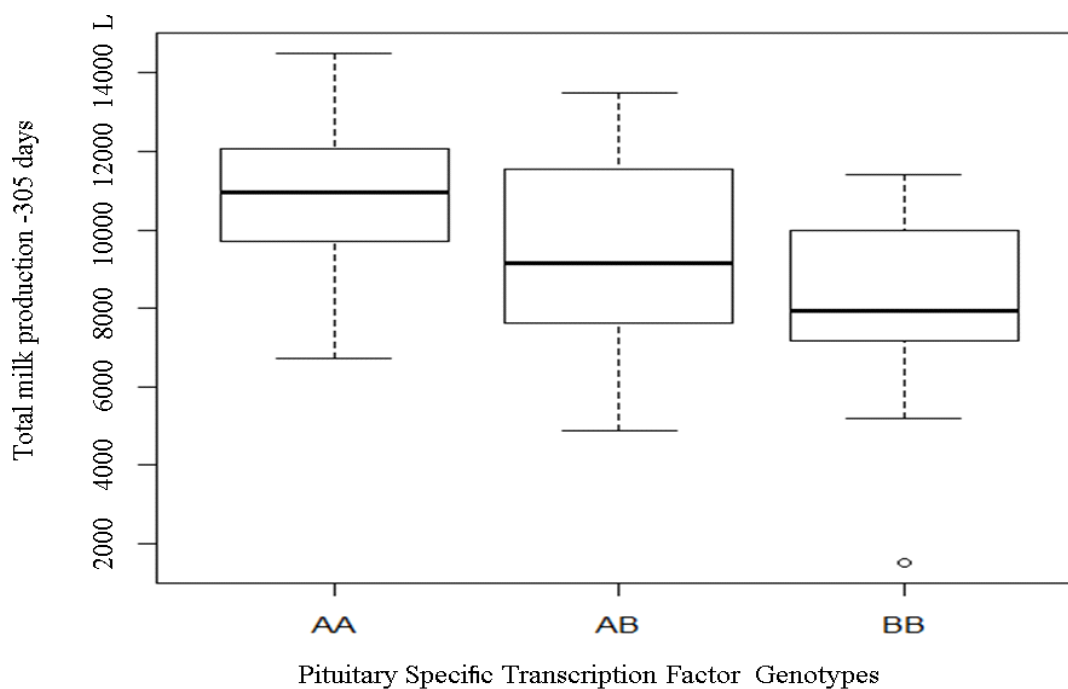
become a popular genetic marker tool commonly used for genetic characterization and identification of possible linkage associations between *PIT-1* gene and milk performance traits. ANOVA analysis revealed that the allelic substitution (AA, AB, BB) effect was significant for milk production with  $p$ -value =  $0.274e-05$  and  $\alpha = 0$  \*\*\*. 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  $\alpha = 0.0$  \*\*\* 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.

### ACKNOWLEDGEMENTS

The authors would like to thank Dr. Robin Abu Ghazaleh and two anonymous reviewers for carefully reading the manuscript, Mrs. Amal Abu Rayan for carrying out the sequencing, and Mrs. Asma Tamimi and Mr. Hasan Altarada for technical support.

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*Full Length Research Paper*

# Application of hazard analysis critical control point (HACCP) system in dairy farms in Khartoum State, Sudan

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Received 16 March, 2014; Accepted 25 April, 2014

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An attempt was made to apply hazard analysis critical control points (HACCP) system in dairy farms in Khartoum State and to suggest control limits for them based on the international standard. World Health Organization (WHO) and Food and Agriculture Organization (FAO) recommended the HACCP system as a modern tool for prevention. Critical points associated with animal health were investigated using screening tests such as California mastitis test (CMT) and rose bengal plate test (RBPT) for detection of bovine mastitis and brucellosis, respectively. The results reveal that the overall prevalence rate of bovine mastitis at the animal level was very high, 69.3% (n=104, out of 150). While, the overall prevalence rate of 36% (n=54, out of 150) was recorded for brucellosis in dairy farms in Khartoum State. Other critical points were obtained from the owners of the dairy farms by means of a questionnaire using non-probability sampling method or willingness of the owners for interview. The main results showed that tick infestation was present in most of the dairy farms, 94% (n=141, out of 150) and application of the odds ratio indicated that tick infestation could be a risk factor (OR=1.694). Using antibiotics for treatment of infected animals was also confirmed in this study as 54.7% (n=82, out of 150). On the other hand, the critical points associated with environment in dairy farms and distribution of the milk in Khartoum State was investigated using questionnaire survey and non probability sampling method was employed. The main results show that the condition of beddings was poor in 105 dairy farms (out of 150), given a percentage of 70%. Absence of the cleaning and disinfection of the teat were recorded in 108 and 104 dairy farms (out of 150), respectively. Many critical points associated with animals or environment as observed in dairy farms in Khartoum State included infectious and zoonotic disease, presence of flies and tick infestation, using antibiotics for treatment or in feed and drinking water and absent of veterinary care in the most dairy farms. While, critical points associated with environment were poor hygienic conditions during different practices, handling, storage and distribution of the milk. Moreover, the control limits for the all mentioned critical points were as follow: access to veterinary service using disinfections and detergents, vectors control, improvement of general hygienic conditions in the environments in the dairy farms during different practice as well as consideration of the issue of temperature during storage and distribution of the milk.

**Key words:** Critical control points, dairy farms, Khartoum State, Sudan.

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## INTRODUCTION

The objective of food safety is to reduce food illnesses and fatalities. The health and hygiene of the cow, the environment in which the cow are housed and milked and hygiene during milking and storage equipments, all influence microbial numbers of milk. Furthermore, milk is considered as good medium for bacteria including pathogenic organisms which have a great impact on public health (Ibtisam and Mohboba, 2007).

Hazard analysis critical control points (HACCP) concept was introduced in the United States in 1971 at the Conference of Food Protection where it was recommended for widespread use (Bauman, 1974). The call for change was galvanized in the early 1990s with a tragic outbreak of *Escherichia coli* O157:H7 food borne illness in the North West of the United States.

Zoonotic diseases such as brucellosis and tuberculosis can be transmitted by using unhygienic milk or milk products. These diseases have been reported from different parts of Sudan. For instance, Khalid (2006) reported that the prevalence rate of brucellosis in Khartoum State was 23.2% using rose bengal plate test (RBPT). A research work by Naglaa (2007) confirmed the presence of tuberculosis in both cattle and man in Khartoum state using single intradermal comparative tuberculin test (SICTT), bacteriological procedures and nested polymerase chain reaction (NPCR).

Mastitis in dairy cows is a multifactorial disease with a long history among the economic loss due to the loss of milk production, treatment costs, extra labor and premature culling of chronically infected cows. Hygiene at all levels: housing, feeding cows in the barn, milking, should be addressed. When an Udder Health Control (UHC) programme is designed and implemented, it warrants a persistent and protocol-based approach by both the farmer (and his co-workers) and a coaching veterinarian in all areas of udder health (Hancock and Dargatz, 1995).

Raw milk is approximately 67-70°C as it comes from the cow, and needs to be chilled to 8°C as fast as possible. To transport fresh raw milk, a cooler or ice chest is needed in order to keep the milk at a cool 40°F or lower at all times. Pre-milking sanitation (udder washing) is a critical point for controlling the bacteria count in milk (Mossel et al., 1995). Hazard-aware dairy workers that work with dairy animals or work in and around dairy operations, should wear personal protective equipment (PPE) for certain jobs and know safety precautions to follow when handling chemicals or when in areas of hazardous atmospheres. Hazard analysis critical control points addresses product quality through the control of the production process. It was originally

developed for the NASA space programme to safeguard astronauts from chemical, physical and micro-biological hazards through food (Hulebak and Schlusser, 2002).

### Objectives of the study

1. To apply hazard analysis critical control points (HACCP) system in dairy farms in Khartoum State in order to clarify if this approach may yield better result than conventional methods.
2. To determine the critical points associated with animals, environment in farms and distribution of the milk which can affect the quality of the milk for human consumption.
3. To suggest the control limits for the different critical points based on the recommended international standards.

## MATERIALS AND METHODS

### Study area

Khartoum State is located in central Sudan, altitude: 382 m (1253 ft). The average temperature in Khartoum, Sudan is 29.8°C (86°F). The average monthly temperature is 10.5°C. The warmest average maximum/high temperature is 42°C (108°F) in May and June. The coolest average minimum/low temperature is 16°C (61°F) in January. Khartoum receives on average 164 mm (6.5 in) of precipitation annually or 14 mm (0.5 in) each month. On balance, there are 18 days annually on which greater than 0.1 mm (0.004 in) of precipitation (rain, sleet, snow or hail) occurs or 2 days on an average month. The month with the driest weather is January, February, March, November and December when on balance 0 mm (0.0 in) of rainfall (precipitation) occurs. The month with the wettest weather is August when on balance 72 mm (2.8 in) of rain, sleet, hail or snow falls across seven days. Mean relative humidity for an average year is recorded as 21.8% and on a monthly basis it ranges from 13% in March, April to 42% in August. Hours of sunshine range between 8.4 h per day in July and 11.1 h per day in February. On balance, there are 3664 sunshine hours annually and approximately 10.0 sunlight hours for each day. On balance there are 0 days annually with measurable frost and in January there are on average 0 days with frost.

### Determination of the critical points

#### *Critical points associated with animals*

Milk samples and serum samples were collected from dairy farms from different sites of Khartoum State (Khartoum, Omdurman and Khartoum North) in order to determine the presence of bovine mastitis and brucellosis.

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**Table 1.** Interpretation of CMT results and relationship with SCC.

Total cell count	Visible reaction	Interpretation	CMT score
0-200,000 0-25% Neutrophil	Milk fluid and normal	Negative	0
150,000-500,000 30%-40% Neutrophil	Slight precipitation	Trace	+
400,000-1,500,000 40%-60% Neutrophil	Distinct precipitation but no gel formation	Weak positive	1
300,000-5,000,000 60%-70% Neutrophil	Mixture thickness with a gel formation	Distinct positive	2
>5,000,000 70-80% Neutrophil	Viscosity greatly increased strong gel that is cohesive with a convex surface	Strong positive	3

Source: Quinn et al. (1994).

### Sampling methods

A total of 150 milk and serum samples were collected from dairy farms (10 farms in Khartoum, 15 farms in Omdurman and 12 farms in Khartoum North). Selection of the dairy farms was based on the willingness of the owners. It means not all the dairy farms have the same opportunity to be selected and this called is non-probability sampling method as described by Thrusfield (2007).

### Collection of the milk samples

A total of 150 milk samples (50 in Khartoum, 50 in Omdurman and 50 in Khartoum North) were collected at the animal level for determination of the presence of bovine mastitis. Before the collection of milk samples, the teats were disinfected with cotton wool moistened with 70% ethyl alcohol. California mastitis test was employed immediately after collection of the milk samples in the dairy farms.

### California mastitis test (CMT)

CMT was carried out using the method described by Schalm et al. (1971) and Quinn et al. (1994). Briefly, equal volumes of commercial CMT reagent and milk sample were mixed and formation of the gel was observed. The interpretation of the result was done as described by Quinn et al. (1994) (Table 1). For the analysis, negative (0) and trace ( $\pm$ ) were considered as negative results and different intensities of positive (1, 2 and 3) were considered as positive results.

### Collection of blood samples

A total of 150 serum samples (50 in Khartoum, 50 in Omdurman and 50 in Khartoum North) were collected for determination of the presence of bovine brucellosis. The blood samples were collected from venipuncture of the jugular vein using syringes. Later sera was separated from the clots, placed in the plastic vials, preserved in ice box and transported as soon as possible to the laboratory, Faculty of Veterinary Medicine, University of Khartoum.

### Rose Bengal plate test (RBPT)

The serum samples and the antigen were removed from the refrigerator and placed at room temperature for an hour, and then

the test was done by dispensing 0.025 ml of each serum to be tested in an enamel plate. The same amount of RBPT antigen was added to each serum and both were mixed together, shaken by hand for four minutes and the test was immediately read. Agglutination appeared as weak positive, positive, strong positive or very strong positive.

### Other critical points that associated with animals

Other critical points associated with animals were obtained from the owners of the dairy farms by the means of questionnaire. A total of 150 owners responded to the questionnaire (50 in Khartoum, 50 in Omdurman and 50 in Khartoum North). Selection of the owners was based on their willingness for interview (Non-probability sampling method) as described by Thrusfield (2007). Information on tick infestation, presence of clinical cases, history of abortion, access to veterinary service, presence of zoonotic diseases and additions of antibiotics to the diet or drinking water were recorded (Appendix 1).

### The critical points associated with environment and distribution of the milk

Information on general conditions of environment in the dairy farms and transportation of the milk were obtained from the owners by the means of questionnaire using non-probability sampling methods as described by Thrusfield (2007). Information such as type of housing, condition of housing, condition of bedding, using detergent, cleaning or disinfection of the teat, milkers, store and distribution of the milk were recorded.

### Data analysis

IBM SPSS version 19 was used for data analysis. The results were presented as descriptive statistic in tables using frequency and percentage. While, analytical statistic using Chi-square (2) and odds ratio (OR) was employed for purpose of getting significance level and estimation of the risk associated with critical points in dairy farms in Khartoum state. For Chi-square (2), the interpretation depended on p-value. For instance, if p-value is less than 0.05, the difference was considered significant. OR was used only for significant association and when the OR is greater than one, the factor could be a risk factor. In contrast, when the OR is less than one, the factor could be protective factor.



**Table 2.** The presence of bovine mastitis and brucellosis in dairy farms in Khartoum State.

Study site	Bovine mastitis		Brucellosis	
	No. of prevalence of examined the cases (%)		No. of prevalence of examined the cases (%)	
1. Khartoum	50	41 27.3	50	15 10
2. Omdurman	50	40 26.7	50	20 13.3
3. Khartoum North	50	23 15.3	50	19 12.7
Over all	150	104 69.3	150	54 36

Bovine mastitis based on CMT and scores of 1, 2 and 3 were considered positive; Brucellosis based on RBPT.

## RESULTS

This study was carried out in dairy farms in Khartoum State to determine the critical points that can influence the quality of the milk as well as to suggest the control limits for them. The presence of bovine mastitis and brucellosis in dairy farms in Khartoum State was investigated and the results revealed that the overall prevalence rate of bovine mastitis based on CMT at animal level was very high, 69.3% ( $n = 104$ , out of 150) 27.3% ( $n=41$ ), 26.7% ( $n=40$ ) and 15.3% ( $n=23$ ), for Khartoum, Omdurman and Khartoum North, respectively. While, the overall prevalence rate of brucellosis based on RBPT was 36% ( $n=54$ , out of 150), 10% ( $n=15$ ), 13.3% ( $n=20$ ) and 12.7% ( $n=19$ ) for Khartoum, Omdurman and Khartoum North, respectively (Table 2).

Other critical control points associated with animals were investigated using questionnaire survey. The main results showed that tick infestation was present in most of the dairy farms in Khartoum state, 94% ( $n=141$  out of 150). Moreover, the presence of the zoonotic diseases as well as using antibiotics for treatment of the infected animals were confirmed in this study as 54.7% ( $n=82$  out of 150) and 75.3% ( $n=113$  out of 150), respectively. It was observed that the access to the veterinary services was absent in most of the dairy farms, given a percentage of 70% ( $n = 105$ , out of 150). The rest of the results are presented in Table 3.

Estimation of some risk factors associated with animals based on access to veterinary services revealed that the tick infestation could be a risk factors ( $OR = 1.694$ ). While using of antibiotics for the treatment of the animals could be a protective factor ( $OR = 0.453$ ) (Table 4).

The control limits of presence of brucellosis and other zoonotic diseases can be achieved by the culling or treatment of the infected animals. Withdrawal period should be considered in case of addition of antibiotics to feed and drinking water or using antibacterial for the treatment of infected animals.

On the other hand, the critical points associated with environment in dairy farms in Khartoum State were investigated using questionnaire survey. The main results showed that the condition of beddings was poor in 105 dairy farms (out of 150) given a percentage of 70%. Absence of the cleaning and disinfection of the teat were recorded in 108 and 104 dairy farms (out of 150) given a

percentage of 72 and 69.3%, respectively. Moreover, hand washing of the milkers was obtained only for 32% of the dairy farms ( $n = 48$ , out of 150). Store of the raw milk at the room temperature was almost found in farms (100%).

Distribution of the milk was dependent on either vehicle without chilling (50%) or donkey cart (49.3%) ( $n = 76$  and 74 out of 150, respectively) (Table 5) (Figures 1 and 2). Estimation of some risk factors associated with environment in the dairy farms in Khartoum State on the basis of regular examination of the milk revealed that hand washing of the milkers could be a risk factor ( $OR = 2.574$ ). While, the condition of the housing (Figure 2) as well as the distribution of the milk could be a protective factor ( $OR = 0.383$  and 0.278, respectively). The results are presented in Table 6.

Good ventilation, avoiding overcrowding and regular removable of waste are known as the best methods for controlling the bad conditions of housing and bedding. While, temperature and good hygienic practice are necessary for protection of raw milk from growth of microorganisms during different practice in the dairy farms: handling, store and distribution of the milk.

## DISCUSSION

This study was conducted in dairy farms in Khartoum State in order to apply HACCP. Both the critical control points associated with animals or environment were determined by using either the screening tests or questionnaire survey.

As seen from the results, the morbidity rate of bovine mastitis and brucellosis was very high in dairy farms in Khartoum State. These diseases are known as one of critical points associated with animals. A number of researchers in Sudan confirmed the presence of infectious disease particularly zoonotic diseases in intensive and semi-intensive production system. For instance, Khalid (2006) reported that the prevalence rate of brucellosis in Khartoum State was 23.2% using Rose Bengal test (RBT).

Similarly, a study by Mahmoud (2010) showed that the prevalence rate of the disease was 24.6, 13.6 and 5% by RBPT, serum agglutination test (SAT), ELIZA and milk ring test (MRT), respectively in West Kordofan State.

**Table 3.** Summary of questionnaire survey responses with regard to critical points associated with animals.

Unit	Site (Khartoum Omdurman Khartoum North)	Total	Chi-square $\chi^2$ p-value	Interpretation
<b>Tick infestation</b>				
Yes	50(100%) 50(100%) 41(82%)	141(94%)	$\chi^2 = 19.1$	Significant
No	0(0%) 0(0%) 9(18%)	9(6%)	$P=0.00$ ( $p<0.05$ )	
<b>Presence of flies</b>				
Yes	35(70%) 38(76%) 28(56%)	101(67.3%)	$\chi^2 = 4.8$	Not significant
No	15(30%) 12(24%) 22(44%)	49(32.7%)	$P=0.09$ ( $p>0.05$ )	
<b>Access to veterinary service</b>				
Yes	11(22%) 16(32%) 18(36%)	45(30%)	$\chi^2 = 2.5$	Not significant
No	39(78%) 34(68%) 32(64%)	105(70%)	$P= 0.3$ ( $p>0.05$ )	
<b>Presence of abortion</b>				
Yes	25(52%) 16(32%) 31(62%)	73(48.7%)	$\chi^2 = 9.3$	Significant
No	24(48%) 34(68%) 19(38%)	77(51.3%)	$P=0.009$ ( $p<0.05$ )	
<b>Presence of zoonotic diseases</b>				
Yes	18(36%) 21(41%) 43(86%)	82(54.7%)	$\chi^2 = 30.1$	Significant
No	32(64%) 29(58%) 7(14%)	68(45.3%)	$P=0.005$ ( $p<0.05$ )	
<b>Using antibiotic for treatment</b>				
Yes	39(78%) 37(74%) 37(74%)	113(75.3%)	$\chi^2 = 0.3$	Not. Significant
No	11(22%) 13(26%) 13(26%)	37(25.7%)	$P= 0.9$ ( $p>0.05$ )	
<b>Addition of antibiotic to feed</b>				
Yes	18(36%) 11(22%) 4(8%)	33(22%)	$\chi^2 = 11.4$	Significant
No	32(64%) 39(78%) 46(92%)	117(78%)	$P= 0.003$ ( $P<0.05$ )	
<b>Addition of antibiotic to drinking water</b>				
Yes	12(24%) 14(28%) 13(28%)	39(26%)	$\chi^2 = 0.2$	Not. significant
No	38(76%) 36(72%) 37(74%)	111(74%)	$P= 0.9$ ( $P>0.05$ )	

Both clinical and sub-clinical mastitis were also observed in dairy farms in Khartoum State (Nuha, 2000; Rofaida, 2010).

Moreover, some pathogens such as *Staphylococcus aureus*, *Streptococcus pyogenes* and *Escherichia coli* are considered to be one of the major pathogens that cause bovine mastitis as well as major public health concern. Many authors have isolated the above mentioned pathogens from different part of the country (Abubaker, 2005; Elias, 2007; Nahid and Ibtisam, 2007). A research work by Naglaa (2007) confirmed the presence of tuberculosis in both cattle and man in Khartoum State using single intradermal comparative tuberculin test (SICTT), bacteriological procedures and nested polymerase chain reaction

(NPCR).

Other information associated with animal health in the dairy farms were obtained from the owners using questionnaire survey and the results confirmed the presence of flies, tick infestation, abortion and zoonotic diseases and there was no access to veterinary services in the most of the dairy farms in the study area. All above mentioned factors have a great impact on animal health which can affect both the quantity and quality of the milk for human consumption.

Regarding the detection of antibiotics in milk, most of the owner in this study used antibiotics for treatment of the animals and some of them added them to feed or drinking water. Similary, Abdel Rahman (2001) stated

**Table 4.** Estimation of some risks associated with animals based on access to veterinary services.

Factor	Chi - square P - value	Odds ratio (OR) 95% CI	Interpretation
Tike infestation	$\chi^2 = 4.103$ $P = 0.043^*$	OR = 1.094 95% CI ( 1.03 - 1.16)	Risk factor
Presence of flies	$\chi^2 = 1.976$ $P = 0.797$	-	Risk cannot be estimated
Presence of abortion	$\chi^2 = 1.221$ $P = 0.269$	-	Risk cannot be estimated
Presence of zoonosis	$\chi^2 = 0.866$ $p = 0.352$	-	Risk cannot be estimated
Using antibiotic for treatment	$\chi^2 = 4.102$ $P = 0.643^*$	OR = 0.453 95%CI(0.21- 0.98)	Protective factor
Addition of antibiotic to feed	$\chi^2 = 1.778$ $P = 0.182$	-	Risk cannot be estimated
Addition of antibiotic to water	$\chi^2 = 0.873$ $P = 0.350$	-	Risk cannot be estimated

P-value was significant ( $p < 0.05$ ); 95% CI = 95% confidence interval.

**Figure 1.** Distribution of the milk using donkey cart.

**Table 5.** Summary of questionnaire survey responses with regard to critical points associated with environment in dairy farms and distribution of the milk.

Unit	Site			Total	Chi-square p-value	Interpretation
	Khartoum	Omdurman	Khartoum North			
<b>Conditions of beddings</b>						
I- Excellent	---			-	$\chi^2=5.14$ $P=0.076 (p > 0.05)$	Not significant
II- Good	12(24%)	12(24%)	21(42%)	45(30%)		
III- Bad	38(76%)	38(76%)	29(58%)	105(70%)		
<b>Conditions Of housing</b>						
I- Excellent	---			-	$\chi^2=14.6$ $P=0.001(p < 0.05)$	Significant
II- Good	26(52%)	26(52%)	42(84%)	94(62.7%)		
III- Bad	24(48%)	24(48%)	8(16%)	56(37.3%)		
<b>Using of detergents</b>						
I- Yes	9(18%)	9(18%)	13(26%)	31(20.7%)	$\chi^2=1.30$ $P=0.522 (p > 0.05)$	Not significant
II- No	41(82%)	41(82%)	32(64%)	119(79.3%)		
<b>Cleaning of the teats</b>						
I. Yes	14(28%)	14(28%)	14(28%)	42(28%)	$\chi^2=0.00$ $P=1.000 (p > 0.05)$	Not significant
II- No	36(72%)	36(72%)	36(72%)	108(72%)		
<b>Disinfection of the teats</b>						
I- Yes	18(36%)	18(36%)	10(20%)	46(30.7%)	$\chi^2=4.01$ $P=0.134(p > 0.05)$	Not significant
II- No	32(64%)	32(64%)	40(80%)	104(69.2%)		
<b>Regular examination of the milk</b>						
I- Yes	25(50%)	37(74%)	18(36%)	80(53.3%)	$\chi^2=14.84$ $P=0.001(p < 0.05)$	Significant
II- No	25(50%)	13(26%)	32(64%)	70(46.7%)		
<b>Hand washing of the milkers</b>						
I- Yes	10(20%)	20(40%)	18(36%)	48(32%)	$\chi^2=5.147$ $P=0.076(p > 0.05)$	Not significant
II- No	40(80%)	30(60%)	32(64%)	102(68%)		
<b>Store of raw milk In the farms</b>						
I- Room temperature	50(100%)	50(100%)	50(100%)	150(100%)	$\chi^2=7.061$ $P=0.029(p < 0.05)$	Significant
II- Refrigerator	0(0%)	0(0%)	0(0%)	0(0%)		
<b>Distribution of the milk</b>						
I- Chilling vehicle	---			-	$\chi^2=5.5$ $P=0.064(p > 0.05)$	Not significant
II- Opened vehicle without chilling	32(64%)	21(42%)	23(46%)	76(50.7%)		
III- Donkey cart	18(36%)	29(58%)	27(54%)	74(49.3%)		

that minimum detectable concentration for oxyteracycline was 2 mg/ml milk, 48 mg/ml milk for benzyl penicillin and 25 mg/ml milk for Tylosin. He also explained that all the milk samples collected from bulk milk of the farms and supermarket were free of antibacterial residues, whereas 76.6% of the samples collected from treated cows with intramammary infusion were positive for antibiotic residues.

Furthermore, Manal (2005) found that 25% of milk samples which were collected from the central market in Khartoum State in summer were positive for antibiotic

residues using the strain *Bacillus subtilis* British type ATCC - bb33 as the test organism and her results revealed high positive samples (37.9%) of antibiotic residues in the winter.

As seen from the results, many critical points associated with the general conditions of the environment in dairy farms were observed. For instance, bad conditions of housing and beddings were recorded for most of the dairy farms. Using detergents, cleaning or disinfection of the teats and hand washing of the milkers were used in small scale in our study area. Moreover,



**Figure 2.** Distribution of the milk using vehicle without chilling.

**Table 6.** Estimation of some risks associated with environment in the dairy farms and distribution of the milk.

Factor	Chi - square P - value	Odds ratio (OR) 95% CI	Interpretation
Conditions of beddings	$\chi^2 = 7.574$ $P = 0.006^*$	OR = 0.385 95% CI (0.191 - 0.765)	Protective factor
Condition Of housing	$\chi^2 = 0.510$ $P = 0.475$	-	Risk cannot be estimated
Using of detergents	$\chi^2 = 1.648$ $P = 0.306$	-	Risk cannot be estimated
Cleaning of the teats	$\chi^2 = 0.021$ $P = 0.884$	-	Risk cannot be estimated
Disinfection of the teats	$\chi^2 = 0.027$ $P = 0.868$	-	Risk cannot be estimated
Hand washing by the milkers	$\chi^2 = 6.741$ $P = 0.009^{**}$	OR = 2.574 95%CI(1.248 - 5.310)	Risk factor
Store of raw the milk	$\chi^2 = 0.602$ $P = 0.43$	-	Risk cannot be estimated
Distribution of the milk	$\chi^2 = 14.254$ $P = 0.000^{**}$	OR = 0.278 95%CI(0.142 - 0.547)	Protective factor

95% CI: 95% confidence interval; Risks was estimated based on regular examination of the milk.

most of the owners did not consider the importance of low temperature with regard to store and distribution of the raw milk. Our findings are in agreement with that of Nahid and Ibtisam (2007) who state that the hygienic quality of the milk that were collected from the super marks in Khartoum State was very low due to the high bacteria counts, isolation of some microorganisms and

detection of *Brucella* by using milk ring test MRT. Cleaning and removal of soil, bedding material and manure from the udder and flanks of the cow before milking is necessary to prevent the entry of many types of bacteria into milk. Milk production in Sudan is faced with several problems such as poor husbandry practices, bad handling of raw milk during its storage at the farm and

during transportation, bad information infrastructure, poor cooling facilities, high ambient temperature and the long distance between sites of production and consumption centers.

An attempt was also made in Sudan to apply HACCP. For example, a study by Nuha (2009) in dairy farms in Khartoum State revealed poor building construction, poor water supply, poor farms as well as milkers hygiene and accumulation of dung and animal waste. The same author stated that two critical points were assigned to milk distribution chain, the first critical point was to control raw milk production hygiene distribution chain, while the second critical control point was to control milk temperature.

### Conclusion

Many critical points associated with animals or environments were observed in dairy farms in Khartoum State: 1, Critical points associated with animals or environments include (infectious and zoonotic disease, presence of flies and tick infestation, using antibiotics for treatment or in feed and drinking water and absent of veterinary care in the most dairy farms). 2, while, critical points associated with environment were poor hygienic conditions during different practices: handling, storage and distribution of the milk.

Moreover, the control limits for the all mentioned critical points were as follow: access to veterinary service using disinfections and detergents, vectors control, improvement of general hygienic conditions in the environments in the dairy farms during different practice as well as the consideration of the issue of the temperature during storage and distribution of the milk.

### Recommendations

1. Dairy farms should implement a documented food safety management system based on HACCP principles.
2. Using bacterial counts or somatic cell count (SCC) as well as regular examination of the milk using screening tests such as CM) and MRT are required for evaluations of the quality of the milk.
3. An attention should be made for increasing awareness of the owners, technical and financial resources, an effective institutional frame work, trained man power and sufficient information on hazard and risks involved.
4. It is important that milk distribution chain should be monitored by health and veterinary authorities to ensure safe milk to consumers.

### Conflict of Interests

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

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