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ACCOMPANIMENT OF THE POLYMORPHISM AT DEFENSIN GENE *LOCI* WITH MILK TRAITS AND MILK SOMATIC CELL COUNT IN EGYPTIAN BUFFALO

By

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ABSTARCT

The aim of this study is to investigate a polymorphism of the defensin gene and search for its association with daily milk traits and milk somatic cell count (SCC) in experimental buffalo population. Composite milk samples were collected monthly from fifty lactating buffaloes and analysed including fat, protein and lactose contents and somatic cell count. Samples were cultured for bacterial presence. The polymerase chain reaction-restriction fragment length polymerase (PCR-RFLP) technique utilizing *Taq*1 endonuclease enzyme was used on genomic DNA extracted from blood samples. Statistical analysis of dataset was performed using the mixed linear model. Within studied buffaloes, no significant associations were found between the RFLP-*Taq*1 and milk yield, fat and protein content and SCC.However, a significant effect was shown of the RFLP-*Taq*1 on the lactose content.

Keywords:

Buffalo population, Defensin gene, Milk traits, Somatic cell count, PCR-RFLP.

INTRODUCTION

In dairy farms worldwide, mastitis is one of the most common and costly diseases approximately70-80% of losses suffered are due to sub-clinical mastitis**(Reneauand Packard, 1991).** The bovine mammary gland is most susceptible to intrammamry infection (IMI) during the periparturient period **(Smith *et al.,* 1985)**.This disease results in marked reduction in the amount of milk synthesized and in changes in levels of specific milk components and in reducing the overall milk quality (**Kitchen, 1981 and Hamann *et al.,* 1994**). Hence, reducing mastitis incidence is important for economic, environmental and welfare reasons. In dairy animals,mammary gland is protected byavariety of defense mechanisms.Most defenses show antimicrobial activity against bacterial and fungal infections (**Selsted *et al.,* 1984 and Lehrer**

***et al.,* 1988).** Although, the mammary gland in dairy animals are characterized by thick keratinized streak canal barrier and strong teat sphincter, effectiveness of those features may be reduced with advanced age thus allowing increased entry of pathogens **(Hamann *et al.,* 1994)**.Despite susceptibility to mastitis is low in buffaloes in comparison with that in cattle**,** poor management conditions may expect a percent increase of subclinical mastitis **(Saleh, 2005).** In dairy livestock, identification of genetic markers related to the immune response, for udder health is more interest **(Ganz,2003).**Therefore, the aims of this work were to find a polymorphism at defensin gene and to explore its relationship with milk traits and SCC in experimental buffalo herds.

MATERIAL AND METHODS

Animals and management:

In the present study, fifty lactating buffalo cows daughters of seventeen sires and eighteen dams raised at the experimental herds belonging to the Animal Production Research Institute, Agricultural Research Center Mehallet Mousa,Kafr El-Sheikh Governorate,and Egypt).

Buffalo cows were housed in semi-open sheds and were kept under the regular systems of feeding and management adopted by APRI. The ration was offered twice daily and clean water was available all the time. Ration given to the animals were determined according to their live body weight and level of milk production. Mineral salt and vitamins were offered regularly. The ration was offered twice daily and clean water was available all the time. Buffalo cows were hand-milked twice daily with recorded the quantity of milk produced by individual buffaloes. Buffalo cows were to be dried two-months before their expected calving dates. Dry off treatment is practiced for lactating buffalo cows. Drugs against diseases and parasites were applied twice a year. Buffalo cows were selected from the first to the ninth lactations.

Milk samples and bacteriological analysis:

The daily milk yield was recorded monthly and fat, protein and lactose were determined on composite milk samples of the two milkings with a MilkoScan (Foss, Hillerφd, Denmark) milk analyzer. Prior to milking, teat ends of studied buffalo cows were cleaned with 70 % ethanol. First streams of foremilk were discarded, and then 15-ml of milk was collected in sterile tubes. Samples were stored at 4ºC until bacteriological analysis. Milk bacteriological test was performed using milk samples by standard method according to American Public Health

Association **(APHA, 1993).**SCC were determined by the fluoro-opto-electronic method using Fossomatic 5000;Foss Electric apparatus,3400 Hillerod, (Denmark).Counting of SCC was adopted within 24h post-collection following the rules of the **International Dairy Federation (1984).** Original scale SCC values were transformed to log arithmetic according to **Ali and Shook (1980)** to meet the characteristics of hypothesis testing. Subclinical mastitis was defined as the presence of *Staphylococcus aureus, Coagulase Negative Staphylococci and Corynbcateria spp* in the same milk sample. Two groups, according to the udder status were established: healthy and infected udder.The following: daily milk yield (MY) and percentages of fat (F %), protein (P %) and lactose (L %) and log SCC were evaluated.

DNA extraction and PCR amplification:

Blood examples (5-mL) were obtained from jugular vein sterilized vacuum tubes (Vacutainer) into K_2EDTA from buffalo cows. The genomic DNA was extracted using the QIAampDNA Mini kit (Qiagen, Germany, GmbH) according to the manufacturer's protocol.

Based on the sequence of the β-defensin 4 (enteric; GenBank no. EF489402), the following primers were designed for amplification of 217-bp gene fragment:F:5'-GATGTCAAAATCAAGTTGAAAAGG-3' and R: 5'- CCTTCCAGTCTTCCTCACAGG -3. Primers were utilized in a 25-µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan),1 µl of each primer of 20 pmol concentration,5.5 µl of water, and 5 µl of DNA template.

The reaction was performed in an applied biosystem 2720 thermal cycler with conditions: primary denaturation 94°C, 4 min; secondary denaturation 94°C, 45 sec; annealing 60°C, 45 sec; extension 72°C, 1 min; 35 cycles, final extension72°C, 10 min. All the amplified DNA fragments were quantified by visualizing it on 0.8% agarose gel under UV lighting.

PCR-RFLP method:

About 10 µl of amplified PCR product was digested with 1µl *Taq*1 endonuclease enzyme using two µL enzyme buffer (10X FastDigest Green buffer), and 17µl water, nuclease-free in reaction tubes. Then the reaction was done at 65°C for 5 min in a thermoshaker (Biometra). The digested products were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1xTBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the funplex PCR products were loaded in each gel slot. A gelpilot 100 bp DNA ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data were analyzed through computer software.

Statistical analysis:

Dataset were statistically analyzed according to the following mixed linear model:

$$y_{ijklmno} = \mu + a_i + h_j + ys_k + l_l + us_m + b\,(X_{ijklmno} - \overline{X}) + g_n + e_{ijklmno}$$

Where, yijklmno is a record of MY or F%, P%, L%, and log SCC of the buffalo cow i; μ is the overall mean; h_j is the effect of herd h (j=1,2 and 3); ys_k is the effect of year-season of calving k (k=1, 2 ,…, 4); l_l is the effect of lactation number l (l =1, 2, …, 9); us_m is the effect of udder status m (m =1 and 2); b is the regression coefficient of studied traits on days in milk; $X_{ijklmno}$ is the days in milk of record ijklmno of the buffalo cow i; $\overline{X}$ is the average days in milk; g_n is the effect of restriction pattern of fragment n (n =1and 2); a_i is the random effect of the buffalo cow i nested within the udder status and restriction pattern of fragment (US.G) and $e_{ijklmno}$ is the random residual effect normally and independently distributed as $(0, I\sigma^2_e)$.

RESULTS AND DISCUSSION

In forty-one of the buffalo cows studied, PCR amplification of the gene encoding β4-defensin resulted one band (217 bp-PCR product), Fig. (1). While in nine buffalo cows, a PCR product did not appear. Actually, two restrictions pattern (uncut 217 bp and 217 and 150 bp) of PCR-RFLP analysis with *Taq*1 endonuclease enzyme, Fig. (2) It was obtained and used for statistical evaluation. The analysis of variance of the fixed effects on studied traits showed a significant effect of the herd on (MY and P%, $p<0.05$). Year-season of calving, udder status and lactation number effects showed a significant effect on (L% and log SCC, $p<0.05$). The effects of days in milk and RFLP pattern of fragments showed no significant effect ($p>0.05$) on all the studied traits except for L%.

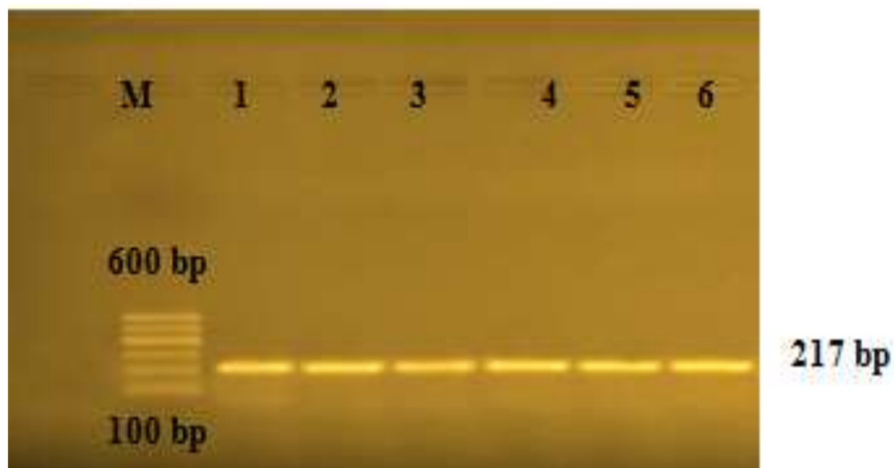

Fig. (1): DNA amplification pattern of β4-defensin gene using a specific primer, M: ladder marker, lanes: 1, 2…, and 6 in studied buffalo cows with healthy or infected udders.

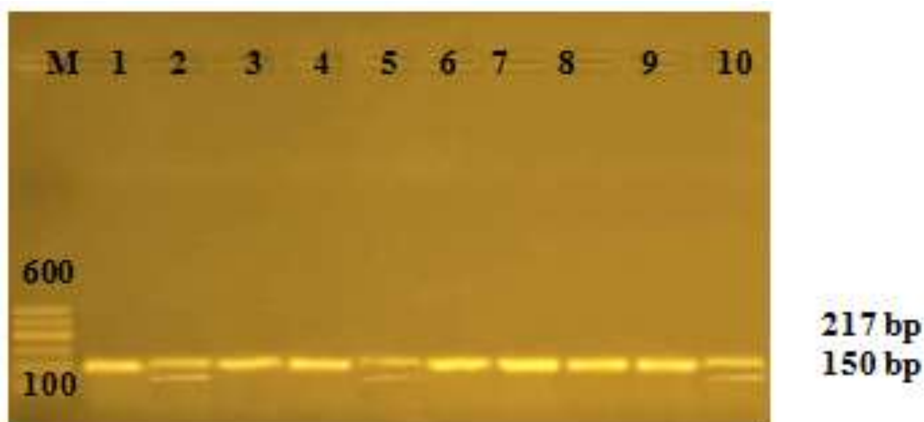

Fig.(2): Restriction fragment patterns of β4-defensin gene after digestion with *Taq*1,M: ladder marker lanes: 1, 3, 4, 6, 7, 8 and 9 (Not digested 217 bp PCR product) and lanes: 2, 5 and 10 (217 and 150 bp) in buffalo cows with healthy or infected udder.

Least-squares means (standard errors) of the investigated traits of RFLP patterns of β4-defensin in studied population with healthy or infected udders are shown in (Tables 1, 2). Buffalo cows with healthy udders and the restriction pattern (uncut 217 bp) did not differ significantly from buffalo cows with the restriction pattern (217 & 150 bp) in MY (*P*= 0.27), F% (*P*= 0. 59), P% (*P*= 0.78) and log SCC (*P*= 0.48). However, there was a significant effect of RFLP-*Taq*1 on the L% (*P* =0.01, Table 1).

Table (1): Least squares means LSM (±standard errors) of investigated traits as affected by RFLP patterns of β4-defensin gene in studied buffalo cows with healthy udders.

	RFLP pattern		
	217 bp (n=16)	217 and 150 bp (n=5)	
Trait	LSM	LSM	*P value*
Daily milk yield, kg	7.09±0.36	6.20±0.73	0.27
Fat content, %	6.10±0.33	5.63±0.66	0.59
Protein content, %	3.31± 0.09	3.26±0.16	0.78
Lactose content, %	5.49±0.02	5.33±0.03	0.01*
Log SCC	4.52±0.08	4.53±0.12	0.48

***Significance level (p <0.05).**

In this study,the RFLP pattern had no significant associations with MY(*P*=0.22),F% (*P*=0.54), P% (*P*=0.12), and log SCC (*P*= 0.36) in studied buffalo cows with infected udders. On the contrary, L% (*P*= 0.01) was significantly affected by the RFLP pattern (Table 2). Generally, the different band patterns that appeared in the studied buffalo were similar to those observed by **Hafez *et al.* (2008)** in Holstein-Friesian cows.

Table (2): Least squares means LSM (±standard errors) of investigated traits as affected by RFLP patterns of β4-defensin gene in studied buffalo cows with infected udders.

	RFLP pattern		
	217 bp (n=14)	217 and 150 bp (n=6)	
Trait	LSM	LSM	*P value*
Daily milk yield, kg	6.69±0.49	6.36±0.60	0.22
Fat content, %	6.28±0.40	6.86±0.50	0.54
Protein content, %	2.80±0.16	3.14±0.13	0.12
Lactose content, %	5.35±0.02	5.16±0.04	0.01*
Log SCC	4.68±0.12	4.62±0.11	0.36

***Significance level (p <0.05).**

Statistical analysis was conducted to explore the associations between RFLP-*Taq*1 and milk characteristics in studied buffalo cows with healthy or infected udders. No significant associations were discovered, in agreement with the results of **(Verma *et al.,* 2009)** in the Indian buffalo and **Souza *et al.* (2015)** on Amazon sheep. However, there has been a significant effect of RFLP-*Taq*1 on the lactose content of buffalo cows with healthy or infected udders.

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