IJAS Iranian Journal of Applied Animal Science

Association of Kappa-Casein Gene Polymorphism with some Biochemical Blood Indicators in Guilan Native Cattle of Iran (*Bos indicus*)

Research Article

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Received on: 25 Feb 2014
Revised on: 11 Jun 2014
Accepted on: 15 Jun 2014
Online Published on: Dec 2014

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Online version is available on: www.ijas.ir

ABSTRACT

Evaluation of the blood and milk biochemical parameters always is considered as effective key factor for animal health and dairy products. In order to assess the association of kappa-casein (K-CN) genetic variants with blood biochemical indicators, blood samples were randomly and individually collected from 126 Iranian Guilan native cattle. Blood plasma was used to measure blood glucose, urea, cholesterol, triglycerides and thyroxine concentrations. The genomic DNA was extracted from the whole blood by the modified Salting Out method. The 350 bp fragment of kappa-casein gene was amplified using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and a pair of specific primers was digested by *HinfI* restriction enzyme. In general, two alleles of A and B with the frequencies of 0.726 and 0.274 and two genotypes of AA and AB with frequencies of 0.452 and 0.548 were detected, respectively. The chi-square test result showed that the studied population was not in Hardy-Weinberg equilibrium. Results of statistical analysis revealed that there was significant difference between glucose, cholesterol and thyroxin levels in both sexes which are associated with the greater levels of testosterone and thyroid hormones in bulls than cows. However, there was no significant difference between males and females for urea and triglyceride levels (P>0.05). There was no significant difference between AA and AB genotypes of kappa-casein (K-CN) gene in Guilan native cattle for the blood parameters measured (P>0.05). According to the current results, kappa casein gene singly cannot be an appropriate marker to study the blood parameters in native cattle of Guilan.

KEY WORDS cholesterol, glucose, PCR-RFLP, thyroxine, triglycerides, urea.

INTRODUCTION

Evaluation of the blood and milk biochemical parameters always is considered as effective key factors for animal health and dairy products (Nozad *et al*. 2012). Normal concentrations of blood parameters considerably are associated with the function of body systems and change in their levels will alter the function of animal body systems. Therefore, using blood serum parameters as a convenient tool to understand the characterization of farm animals has been recommanded (Qureshi, 1998). Today, molecular markers can be an appropriate tool in selection programs of farm animals. The kappa-casein is one of the proteins which are expressed in milk and its polymorphism may be act as an informative molecular marker for yield and composition of milk and blood biochemical parameters (Oprzadek *et al*.

1999). Blood biochemical parameters can be easily measured at different stages of growth in animals. Using biochemical indicators which are not sex-limited and can be measured at different stages of an animal's age may be particularly successful in improvement of dairy cattle. Therefore, the application of such markers can enhance the genetic progress and reduce the cost on identification of genetically superior individuals (Bittante *et al*. 1987). Bovine kappa-casein is 19000 KDa peptide which located on chromosome 6.

The kappa-casein variants A and B differ in the structure of amino acids 136 and 148. In position 136, threonine (ACC) is replaced by isoleucine (ATC) and in position 148, aspartic acid (GTA) is replaced by alanine (GCT) (Lin *et al*. 1992). Several researches on different breeds of dairy cows indicated that there is a significant relationship between the kappa-casein genetic variants and milk yield, milk fat and protein percentages. Bovenhius *et al*. (1992) reported that cows with BB genotype of Kappa casein produced less milk and more milk protein compared to cows with AA genotype.

Although many studies have been conducted on relationship between kappa-casein gene polymorphism and production traits, but there is few information about its relationship with blood parameters (Galila *et al*. 2008; Buchberger and Dove, 2000; Alipanah *et al*. 2007). In some investigations, often it has been observed an association of multiple loci and epistasis effects from several genes on some blood parameters such as glucose, urea, cholesterol, triglycerides and thyroxin excluding insulin and urea (Oprzadek *et al*. 1999). Considering the importance of blood parameter concentrations in animal health and production and also the association between the milk casein concentrations and processed dairy products, the aim of this study was to evaluate the association between kappa-casein gene polymorphism and some biochemical blood indicators in Guilan native cattle of Iran.

MATERIALS AND METHODS

A total of 126 Guilan native cattle consisted of 70 cows and 56 bulls belonging to the Agriculture and Natural Resources Research Center, Fouman native cattle breeding center and the census herds in Guilan province of Iran were selected which were similar in age and nutritional conditions. Whole blood samples were collected twice with an interval of one week between them from the jugular vein of animals prior to the morning feeding (after 24 hours starvation). The samples were transported immediately to the laboratory. Two sets of blood samples (2×4 mL) were obtained at the first sampling which one set was stored in a freezer at -20 ˚C for further DNA extraction and for the second set, plasma was separated immediately by centrifugation (5 min at 5000×g) and stored at -20 ˚C until analysis for glucose, urea, cholesterol, triglycerides and thyroxine concentrations. Plasma glucose, urea, cholesterol and triglycerides were measured by spectrophotometric method using commercial kits (Pars Azmun, Tehran, Iran) and thyroxin concentration was measured by ELISA using commercial kit (Pishtaz Teb, Tehran, Iran). In order to measure blood parameters, each sample was observed three times which the mean number of repetitions was recorded as the final concentration. Genomic DNA was extracted from blood leukocyte cells using the modified Salting Out method (Javanrouh *et al*. 2006). Quality and quantity of extracted samples was assessed by electrophoresis on 0.8% agarose gel and using spectrophotometry method. A 350 bp fragment from exon 4 of kappa-casein gene was amplified using PCR-RFLP and a pair of specific primers suggested by Medrano *et al*. (1990). The primer sequences used for the amplification of κ-casein were as follows:

5`-ATC ATT TAT GGC CAT TCC ACC AAA G-3` (forward) and 5`-GCC CAT TTC GCC TTC TCT GTA ACA GA-3' (reverse).

The PCR amplification reactions were used as follows: initial denaturation at 94 ˚C for 3 min, followed by 32 cycles of denaturation at 94 ˚C for 30 s then annealing at 60 ˚C for 30 s and extension at 72 ˚C for 30 s and final extension at 72 ˚C for 3 min. The PCR was carried out into 25 µL final volume containing 100 ng DNA template, 1 × PCR Buffer, 10 pmol of each primer, 100 µ*M* dNTP Mix, 1.5 m*M* $MgCl_2$, 1 unit of Taq DNA polymerase and ddH_2O. The PCR products were separated and recognized by electrophoresis on 1.5% agarose gel stained with etithium bromide. Also, DNA marker was used to confirm the desired PCR products length.

In order to study the kappa-casein gene polymorphism, 15 µL of the PCR products was digested with 6 U of *HinfI* . Samples were incubated at 37 ˚C for 3 h. The visualization of digestion products was performed in horizontal electrophoresis (70 volts, 110 min) of 2.5% agarose gels stained with etithium bromide and digested fragments size was recognized by pBR322 / BsuRI DNA markers and genotypes were determined based on the difference in fragments' size on the gel. In order to estimate the allelic and genotypic frequencies, expected and observed heterozygous, Nei heterozygosity and Chi-square test ($\chi 2$) were performed using Pop Gene V. 1.32.

Also, the polymorphic information content (PIC) in the kappa casein gene locus for studied population was estimated using HET 1.8 software. Association between the kappa-casein genotypes and blood parameters were ana-

lyzed according to the following statistical model using (SAS, 1996) program:

$Y_{ikno} = \mu + Sex_i + SN_k + b(Age_{ikno} - \overline{Age}) + CSN3_n + e_{ikno}$

Where:

Y_{ikno}: observed value for studied traits.

μ: the overall mean of population for different traits.

Sex_i: fixed effect of animal sex (i=1, 2).

SN_k: fixed effect of blood sampling (k=1, 2).

$b(Age_{ikno} - \overline{Age})$: covariate effect of age at sampling time to the number of same age individuals which were sampled.

$CSN3_n$: fixed effect of kappa casein genotype.

e_{ikno}: random residual error.

RESULTS AND DISCUSSION

Analysis of blood parameter concentrations showed that all data has normal distribution and the normal range was also excluded. The quality and quantity of extracted DNA was estimated using a defined standard DNA (λ DNA) on agarose gel. Agarose gel analysis of the DNA resulting from extractions indicated the presence of single band with high intensity in most cases, although there were some samples with poor purity and smears which were re-extracted and also high concentration samples were diluted with distilled water again to prepare appropriate quality of extracted DNA for PCR. Some extracted genomic DNA samples that were loaded on 1% agarose gel are shown in Figure 1.

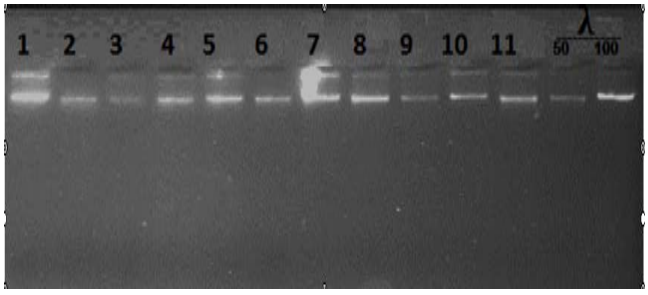

Figure 1 DNA extracted from blood of Guilan native cattle of Iran

Electrophoresis of PCR products on 1.5% agarose gel showed that designed primers acted well and produced specific fragments for the kappa-casein gene with 350 bp in length. The digestion results of the kappa-casein gene amplification products indicated polymorphism in Guilan native cattle. An allele in the KCN- *HinfI* locus had two restriction sites caused three fragments (84, 132 and 134 bp) while there was a restriction site for *HinfI* endonuclease for B allele that exhibited two fragments (84 and 266 bp) due to the substitution of cytosine (C) to adenine (A) nucleotide in position 5345 (exon 4) of KCN locus that alters the restriction site sequence of *HinfI* (Figure 2).

Allelic and genotypic frequencies for the HinfI-RFLP polymorphism of kappa-casein gene are shown in Table 1. Results indicated that A allele frequency (0.726) was higher than B allele (0.274) and the greatest genotypic frequency was for AB (0.545) in the studied population.

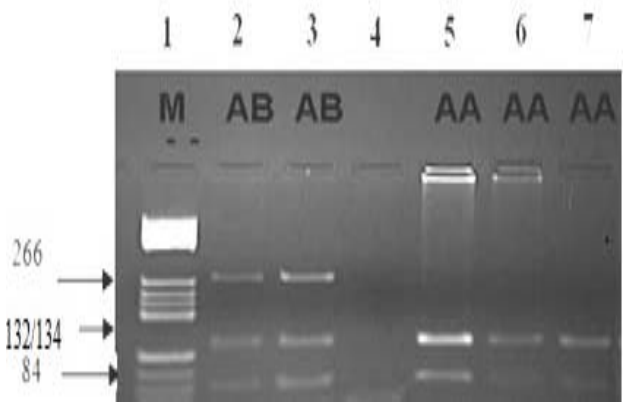

Figure 2 Results of the kappa-casein gene PCR products digested by *HinfI* on 2.5% agarose gel, staind dy ethidium bromide, Lane 1 is a DNA size marker (pB322/BsuRI), Lanes 2 and 3 are AB genotype (84132/134 and 266 bp), Lane 5, 6 and 7 is AA genotype (84 and 132/134 bp)

Table 1 Frequencies of genotypes and alleles observed in the kappa-casein gene (*HinfI*-RFLP) and $\chi 2$ test.

	No. of cows	Allele		Genotype		χ^2
		A	B	AA	AB	
Frequen	126	0.726	0.274	0.452	0.548	17.609
±SE	-	0.016	0.016	0.031	0.031	-

χ^2: chi-square test.
SE: standard error.
** (P<0.01).

The results of current study were consistent with Alipanah *et al.* (2005) that reported the greater frequency of A allele than B allele in Black and Red Pied Cattle. Absence of BB genotype in the studied population is probably due to small sample size and the low frequency of B allele. Also, Galila *et al.* (2008) in a review on buffalo and Holstein cows for the genetic analysis of the variants of kappa-casein gene using PCR-RFLP method, genotyped 20 Holstein cows and found 17 AA genotypes and 3 AB genotypes while BB genotype was not observed in these samples. These studies confirmed the results obtained in the present study for Guilan native cattle. The observed genetic diversity was similar to other reports on Polish (Zwierzchowski *et al.* 1995) and Friesian cows (Klauzinska *et al.* 2000). The $\chi 2$ test (17.609<6.635) confirmed significant departure from Hardy-Weinberg equilibrium (P<0.01) in the studied population.

It can be due to occurrence of several migrations, selective breeding, gene drift and mutation which had significant effect in small populations. The results of this study showed that the observed heterozygosity (0.548) is relatively higher than that expected (0.399); therefore, it can be concluded

that the inbreeding is low and genetic diversity is rather high in the studied population. Also, rather high Shannon's Information index (I) and expected heterozygosity (Nei's) showed high genetic diversity in the studied population.

The observed and effective numbers of allele's are criteria for determining the polymorphic loci. Genetic variation and heterozygosity statistics for the kappa-casein gene locus in Guilan native cattle are shown in Table 1.

Table 2 Genetic variation and heterozygosity statistics for the kappa-casein gene locus in Guilan native cattle

	na	ne	Hom_e	H_e	I	PIC	N_{ei}
K-CN locus	2	1.66	0.601	0.399	0.587	0.373	0.398
±SE	-	0.039	0.0321	0.013	0.015	0.008	0.014

Na: observed number of alleles; ne: effective number of alleles; Hom_e: expected homozygosty; H_e: expected heterozygosity; I: shannon's information index; PIC: polymorphic information content and N_{ei}: expected heterozygosity.
K-CN: kappa-casein.
SE: standard error.

Summary statistics of glucose, cholesterol, thyroxin, triglycerides and urea in males and females are presented in Table 3. According to these results, minimum measured levels of glucose, urea, cholesterol, triglycerides, and thyroxin were 57.5, 14.605, 181.25, 175 (mg/dL) and 3.25 (µg/dL), respectively and the maximum ones were 113.25, 37.5, 338.889, 247.5 (mg/dL) and 21(µg/dL), respectively. Glucose, cholesterol, triglycerides, urea and thyroxin mean on measuring has been gained 86.046, 279.271, 237.638, 20.219 (mg/dL) and 7.895 (µg/dL), respectively. The results of analysis of biochemical indicators in blood plasma of Guilan native cattle are given in Table 4.

Results of statistical analysis revealed that there was significant difference in glucose, cholesterol and thyroxin levels between sexes which are probably associated with the greater levels of testosterone and thyroid hormones in bulls than cows. However, there was no significant difference between both sexes for urea and triglyceride levels ($P>0.05$).

Nozad *et al*. (2011) studied the relationship between blood biochemical indicator concentrations with the quality and quantity of milk in Holstein dairy cows. They reported that mean urea in high and low producer cows were 26.5 and 23.6 (mg/dL), respectively, but it seems that Guilan native cattle had lower concentration of urea in both sexes than Holstein cows.

Also, Nozad *et al*. (2011) reported that averages of blood cholesterol content in high and low producer cows were 162.6 and 192.9 (mg/dL), respectively, which was lower than that of Guilan native cattle. Therefore, it might relate to the higher milk fat percentage. Blood triglyceride concentrations in Holstein cows also had markedly difference with Guilan native cattle in our study which it can be justified according to the relationship between cholesterol and triglyceride. In a similar study on crossbred cows in Pakistan, Ijaz *et al*. (2004) reported that blood levels of glucose, cholesterol and urea were 50.72, 199.12 and 30.88 (mg/dL), respectively, whereas urea concentration was greater than that of Guilan native cattle but glucose and cholesterol concentrations were lower than those of current study. In general, concentrations of hormone and metabolites were in the range of values reported by other researchers (Schams *et al*. 1991; Graml *et al*. 1995).

Least squares means of biochemical blood indicators for different genotypes and sexes are shown in Table 4. The comparison of least squares means of homozygous (AA) and heterozygous (AB) genotypes in each of two sexes showed no significant differences for glucose, cholesterol and thyroxin between two genotypes in Guilan native cattle, whereas there was significant difference between homozygous (AA) and heterozygous (AB) genotypes of both sexes for glucose, cholesterol and thyroxin. Oprzadek *et al*. (2003) evaluated associations between genetic variants in the three loci (growth hormone (GH), kappa-casein (CSN3) and β-lactoglobulin (LGB) and concentrations of triiodothyronine (T3), thyroxine (T4), insulin (Ins), glucose (Glu), urea (Ur), creatinine (Cr), cholesterol (Chol), as well as the activity of alkaline phosphatase (AP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). They reported that only the levels of Ins and Ur were not related to the loci considered.

The level of T3 was affected by the interaction between the GH and LGB loci, while that of T4 by the epistasis of GH, CSN3 and LGB. The Cr and Chol levels depended on CSN3 and LGB as well as their interaction. GH×CSN3 loci were found involved in the AP, all the three loci in ALT, and GH × LGB in AST activity molding.

The T3 range was significantly affected by the interaction between GH and LGB loci, while T4 range affected by interaction of CSN3 with the GH and LGB. Glucose level depended on LGB, due to the dominance effect at that locus.

Urea level was not related to the considered genotypes, while Cr and Chol levels depended on both CSN3and LGB as well as their interaction (Oprzadek *et al*. 1999). It was emphasized, however, that differences in metabolite concentrations due to breeding value of animals are often small, not significant, and frequently not repeatable (Akers, 2000; Woolliams and Lovendhal, 1991). There are significant differences in milk yield merit of young calves for blood growth hormone, insulin and glucose levels which all of these were greater in animals with high *vs*. low milk yield merit.

Similar differences for glucose and insulin were reported, but no differences were found for urea, creatinine and free fatty acids between calves with high and low-breeding indices (Min *et al*. 1993).

Table 3 Descriptive statistics for biochemical indicators of blood plasma of Guilan native cattle

Biochemical indicators	Sex	Mean	Minimum	Maximum	Total mean	SD	±SE
Glucose (mg/dL)	Male	90.138	73.25	106.25	86.041	9.966	0.888
	Female	82.764	57.5	113.25			
Cholesterol (mg/dL)	Male	287.537	241.667	338.889	279.271	32.293	2.877
	Female	272.659	181.25	334.722			
Triglycerides (mg/dL)	Male	238.929	212.5	247.5	237.638	11.069	0.986
	Female	236.607	175	247.5			
Urea (mg/dL)	Male	20.316	14.605	25.789	20.219	2.937	0.262
	Female	20.099	15.789	37.5			
Thyroxine (µg/dL)	Male	9.205	3.5	21	7.895	4.228	0.377
	Female	6.847	3.25	20			

SD: standard deviation.
SE: standard error.

Table 4 The least square means and their standard errors for blood parameters in different genotypes and sexes of native cattle in Guilan

Sex	Genotype	Blood Parameter	Least squares means	±SE	P-value
F	AA	Glucose	82.57	1.62	< 0.0001
		Cholesterol	270.45	5.53	< 0.0001
		Triglyceride	238.8	1.92	< 0.0001
		BUN	20.48	0.52	< 0.0001
		Thyroxin	6.52	0.71	< 0.0001
	AB	Glucose	82.94	1.54	< 0.0001
		Cholestrol	274.62	5.22	< 0.0001
		Triglyceride	234.93	1.81	< 0.0001
		BUN	20.17	0.49	< 0.0001
		Thyroxin	7.13	0.67	< 0.0001
M	AA	Glucose	90.04	1.91	< 0.0001
		Cholestrol	288.77	6.48	< 0.0001
		Triglyceride	240.42	2.25	< 0.0001
		BUN	20.33	0.6	< 0.0001
		Thyroxin	8.66	0.84	< 0.0001
	AB	Glucose	90.21	1.66	< 0.0001
		Cholestrol	286.61	5.62	< 0.0001
		Triglyceride	273.81	1.85	< 0.0001
		BUN	19.99	0.52	< 0.0001
		Thyroxin	9.61	0.72	< 0.0001

F: female and M: male.
SE: standard error.

Likely, kappa casein gene polymorphism alone cannot affect the concentrations of glucose, cholesterol and thyroxin but it can be effective as epistasis with other loci, whereas the sex effect was significant because it can be associated with the greater levels of testosterone and thyroid hormones in bulls than cows and also its effect on the concentration of other hormones and particularly steroid hormones and very prominent role of these hormones in increasing metabolite concentrations.

Also, the sex effect can be justified by remarkable increases in metabolic rate and capacity in the males than females. In any cases, it should be noted that conflicting and disappointing results will often be obtained about determination of blood plasma and serum composition. These differences may be affected by environmental factors and animal selection. Reports on hormones and metabolites as indicators of dairy merit were presented by Robinson *et al*. (1992).

There was no significant difference between homozygous (AA) and heterozygous (AB) genotypes in each of two sexes and between both sexes for triglyceride and urea concentration.

Concentrations of blood triglyceride and urea are probably affected by other gene loci or epistasis between multiple loci and kappa-casein gene polymorphism alone had no significant effect on the concentrations of these two parameters.

CONCLUSION

According to observations and results of statistical analysis of kappa casein gene polymorphism in the current study, this gene singly cannot affect the blood parameters concentrations and in spite of significant differences between sexes cannot be an appropriate marker to study the blood parameter concentrations of native cattle in Guilan.

ACKNOWLEDGEMENT

The authors sincerely thank the university of Guilan, Department of Veterinary Medicine, Guilan Agriculture and Natural Research Center and Jihad- Agricultural Organization in Guilan for providing research facilities.

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