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MOLECULAR MARKER ASSISTED STUDY OF KAPPA-CASEIN GENE IN NILI-RAVI (BUFFALO) BREED OF PAKISTAN

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ABSTRACT

The present study was conducted to investigate the existence of polymorphism at Kappa-casein (κ-CN) locus in the Nili-Ravi buffalo. A PCR-RFLP method was used to distinguish buffalo κ-CN alleles. The genotypes were confirmed through specific, clearly distinguishable DNA band pattern resulting from digestion with three selected restriction enzymes (*Hinf*I, *Hae*III and *Mae*II). Analysis of 163 animals revealed that all animals were monomorphic, showing only BB genotype. It was concluded that κ-CN B allele, which has significant effect on milk quality reported previously, may be included in breeding strategies of dairy animals. The monomorphism for κ-CN locus observed in buffalo population can lead to the development of a method to identify mixtures of cow and buffalo milk in cheese processing.

Key words: Genotyping, κ-CN, milk protein polymorphism, PCR- RFLP.

INTRODUCTION

Livestock is a major financial factor in the economy of Pakistan, accounting for 52.2% of agriculture added and about 11% of GDP. Pakistan, being the fifth largest milk producing country of the world, has an annual gross milk production of about 42,199 million tons, with buffalo contributing 71% of the total milk production. Pakistan possesses 29 million heads of buffalo (GOP, 2007-08). In this context, the most important dairy breed is Nili-Ravi which is the most adaptable and versatile of all the domesticated animals in Asia. The accurate selection still carries an extreme importance to bring stable improvement in milk quantity and quality.

Selection on the basis of molecular markers is more reliable than any other criterion. Adaptation of such a selection depends on the identification of candidate genes by determining the co-relationship between physiological or biochemical processes and the trait. Polymorphism of such genes has great potential to be used as unique genetic marker. Since centuries, milk quality and quantity have always been taken as traits of supreme interest. Variations in composition and production of milk due to certain genetic (involving many genes) and environmental factors such as climate, management and stage of lactation make it a multi factorial polygenic trait (Henderson, 1971).

Milk proteins polymorphism has attracted intensive research interest due to its importance in breed selection. Different genomic variation in the κ-CN locus has been strongly associated with differences in milk composition, its processing properties and dairy products. Such a variation can be detected by restriction analysis through electrophoresis. Some other variations named as post transcriptional variations are also seen in casein molecule (Mclean, 1987; Kemenes *et al*., 1999). Restricted expression of casein during lactation limits the use of milk protein analysis for genotypic evaluation but DNA based genotyping through PCR-RFLP has made this evaluation possible as well as simple for a large number of animals. Adopting such an evaluation method broadens the selection process and has influence regardless of sex, age or physiological stage of the animal. This also leads to an improved response to selection in relatively much lesser time (Madrano and Aguilar-Cordova, 1990).

Bovine casein is encoded by a 200 kb DNA fragment located at chromosome No. 6 arranged in the order of αS1, αS2, β and κ. κ-CN fragment spans the 13 kb DNA sequence divided into five exons and intervening sequences and constitutes about 25% of the casein fraction (Lien and Rogne, 1993).

κ-CN has been extensively studied for its role in stabilizing the casein micelles and its influence on the manufacturing properties of milk. For several breeds, the genetic variability in the κ-CN locus has been reported each with a different allelic frequency based on genetic diversity among breeds. Various allelic variants have been described for κ-CN gene in different cattle breeds, which include A, B, C, E, F, G, H, I and AI (See review by Soria *et al*., 2003). Among these, variants A and B are most commonly found and variant B is predominantly concerned with processing properties of milk and has better lactodynographic properties (Lin *et al*., 1992). In variant B, due to a single base mutation in the κ-CN locus, isoleucine substitutes threonine and aspartic acid is substituted by alanine (Pinder *et al.,* 1991).

Keeping in view the importance of milk quality in the economy of Pakistan and to study the genetic variability among buffalo breeds, the present study was designed to optimize a standard molecular method at DNA level and to identify the occurrence of polymorphism at κ-CN locus in indigenous Nili-Ravi buffalo breed.

MATERIALS AND METHODS

Blood sample collection

A total of 163 Nili-Ravi buffaloes were sampled during their first lactation, both from Government as well as private farms, maintained at standard balanced diet. About 5 ml blood was collected in K_3-EDTA coated sterile vaccutainers and stored at -20^{0}C until used for DNA extraction.

DNA extraction

Genomic DNA was extracted from leukocytes according to the procedure described by Sambrook *et al*. (1989). The quality and concentration of extracted DNA was assessed by visualizing it on 0.8% agarose gel under UV light.

Polymerase Chain Reaction

The primers used for the amplification of the κ-CN gene fragments were already reported by Barroso *et al*. (1998) with the following nucleotide sequence: KF 5′-TGTGCTGAGTAGGTATCCTAGTTATGG-3′ KR 5′-GCGTTGTCTTCTTTGATGTCTCCT-3′.
Amplification reaction was done in a final volume of 25 µl, containing 100 ng DNA, 50 pico moles of each primer, 1X Taq polymerase buffer (10 mM Tris HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.5U Taq DNA polymerase. The amplifi- cation was carried out in thermal cycler with the following amplification conditions: 94°C for 5 min (initial denaturation), 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min with a final extension step of 72°C for 10 min upto 35 cycles.

Positive and negative PCR controls

Reliability of the PCR products was further confirmed through both positive and negative controls. Positive control was the PCR product resulting from amplification of an extracted DNA sample with the same set of primers and same procedure was repeated thrice. Negative control having no DNA template was used to check presence of any contamination in the PCR reagents.

Restriction Fragment Length Polymorphism (RFLP) technique

PCR products were subjected to digestion by restriction enzymes. In reaction with a total volume of 20µl, 10µl of the amplified fragment was digested separately with 5U each of *Hae*III, *Mae*II and *Hinf*I in three separate reaction tubes. After incubation of the reaction mixture at 37^{0}C for 12-16 hrs, the digested fragments were subjected to electrophoresis on 2% agarose gel stained with ethidium bromide in 1X TBE as the running buffer at 60V for approximately 120/min. Visualization of the restricted DNA bands was done under UV light. Size of different DNA bands resulting from restriction with each enzyme was confirmed through standard DNA marker and the size of unrestricted PCR product was taken as control.

RESULTS

Analysis of PCR Amplified κ-CN gene fragment

PCR amplification using primers KF and KR yielded a 453 bp DNA fragment of κ-CN gene. Presence of a single band visible under UV light removed the need for PCR purification step before restriction analysis (Fig. 1).

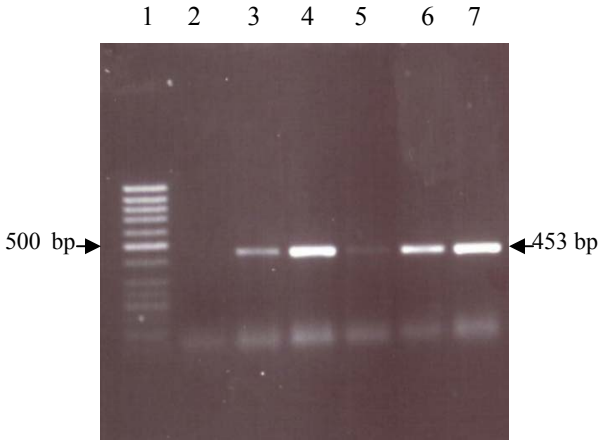

Fig. 1: Analysis of amplified product of κ-CN gene (Exon IV) on 1.8% agarose gel. Lane 1: 50 bp DNA ladder (MBI, Fermentas). Lane 2: Negative control. Lane 3: Positive control. Lane 4-7: Amplified product of bovine κ-CN gene which resulted 453 bp bands.

RFLP analysis of amplified DNA fragment of κ-CN gene

Amplified products from Nili-Ravi buffalo, after being digested with *Hinf*I, generated two DNA fragments (426 and 27 bps). Digestion of the same PCR product with each of *Hae*III and *Mae*II yielded two separate DNA fragments of different sizes i.e. 223, 230 bps and 254, 199 bp, respectively (Fig. 2). Cumulative analysis of the results depicted the existence of only B allele.

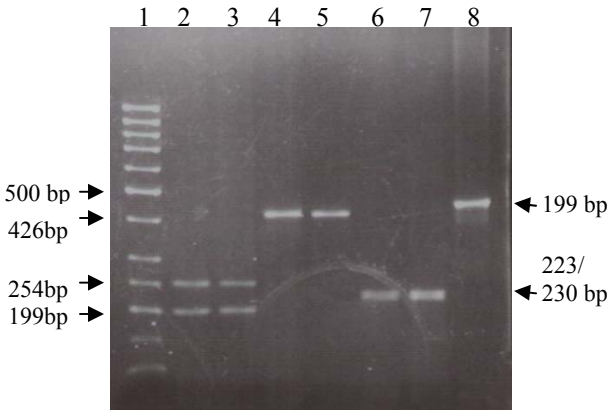

Fig. 2: Restriction analysis of amplified product of κ-CN gene (Exon IV) with *Mae*II, *Hinf*I and *Hae*III on 2% agarose gel electrophoresis. Lane 1: 50 bp DNA ladder (MBI, Fermentas). Lane 2-3: digested PCR product with *Mae*II, Lane 4-5 with *Hinf*I, Lane 6-7: *Hae*III restriction endonucleases.

DISCUSSION

Restriction pattern observed following enzymatic digestion of 453 bp amplified fragment of κ-CN gene was compared with already know patterns (Table 1) specific for each genotype. Restriction analysis of κ-CN amplified fragment with *Hinf*I, *HaeIII* and *MaeII* generated different DNA length fragments of 426 and 27 bp, 223 and 230 bp, 554 and 199 bp, respectively specific for BB genotype. Mitra *et al*. (1998), Pipalia *et al*. (2001) and Ontaviano *et al*. (2005) also found monomorphism (BB) for this gene in buffalos. However, Sing *et al*. (2005) found two alleles A and B for κ-CN locus in Murrah and Bhadawari breeds but they have reported monomorphism in Surti and Mehsana breeds of buffalo. Similarly, two types of alleles were found in Murrah, Surti and Pandharpuri breeds of buffalo by Patel *et al*. (2007). Monomorphic form BB of κ-CN is responsible for higher yield in cheese making as well as milk and milk protein yield (McLean, 1987). The cheese production can be increased by 10% if milk from cows of genotype BB of κ-CN is used (Marziali and Ng-Kwai-Hang, 1986).

Our results clearly indicate uniform and homozygous population of Nili-Ravi buffalos for κ-CN B allele. The present study is the first report on κ-CN genotyping of Nili-Ravi buffalo of Pakistan. This study shows that the PCR-RFLP test is quite easy and rapid method of genotyping bovine milk protein loci and the results of this study can also be helpful to study the κ-CN gene polymorphism in other bovine breeds in Pakistan. So, the application of molecular marker in conjunction with conventional animal breeding practices can be useful for improvement in animal selection and identification of best alleles to utilize in breed selection.

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Table 1: DNA fragment sizes for each genotype for κ-CN locus after digestion with *Hinf* I, *Hae* III and *Mae* II: Reviewed in Barroso *et al.* (1998)

	Restriction enzyme										
Genotypes	***Hinf* I**			***Hae* III**					***Mae* II**		
	Sizes in base pairs (bps)										
AA	-----	326	100	27	230	223	-----	-----	-----	254	199
AB	426	326	100	27	230	223	-----	-----	-----	254	199
BB*	426	-----	-----	27	230	223	-----	-----	-----	254	199

* Genotype found in our study.

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