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Full Length Research Paper

Calpastatin (*CAST*) gene polymorphism in Kajli, Lohi and Thalli sheep breeds

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Calpastatin-encoding gene (*CAST*) is located on the fifth chromosome of sheep and it plays an important role in the development of muscles and in meat tenderness. The present study was conducted to investigate a calpastatin (*CAST*) gene polymorphism in Pakistani Thalli, Lohi and Kajli sheep breed. Random blood samples were collected from 300 animals (100 samples from each Thalli, Lohi and Kajli breeds). Genomic DNA was extracted using phenol-chloroform extraction method. A 622 bp *CAST* gene segment (exon 1C/1D region) was amplified by polymerase chain reaction (PCR) using ovine specific primers. Restriction fragment length polymorphisms (RFLPs) in the amplified fragments were studied using *Msp*1 restriction enzyme. Frequencies of MM, MN and NN genotypes were found to be 77, 20 and 3% in Lohi breed and 68, 26 and 6% in Kajli breed respectively. In Thalli sheep, only the MM (80%) and MN (20%) genotypes were detected. Chi-Square test ($p < 0.05$) showed that all the three populations used in this study were in Hardy-Weinberg equilibrium. By comparing the results of this study with those of previous studies, it seems that the MM genotype is the dominant genotype and the M allele is the dominant allele in small ruminant breeds belonging to different geographical locations.

Key words: Thalli, Lohi, *CAST* gene, Kajli, polymorphism, *Msp*1, PCR-RFLP.

INTRODUCTION

Improvement of meat quality is one of the top priorities of the beef industry and meat tenderness is a primary characteristic for consumers' acceptance (Morgan et al., 1991). The problem of producing inconsistently tender meat needs resolution. Attempting to resolve the problem of variability of meat tenderness, many reports on the inheritance of meat tenderness traits, such as calpastatin activity, have been published (Marshall, 1994; Bertrand et al., 2001; Riley et al., 2003). Sheep breeds can be classified into two main categories thin-tailed (Kajli, Lohi, and Thalli etc) and fat-tailed (Gojal and Harnai etc) used for the production of best quality wool and mutton (Khan et al., 2007). There are 28 sheep breeds of Pakistan and the most important one is Lohi breed, which belongs to the irrigated areas of the central Punjab (Babar, 2008; Al-Shorepy, 2001). Another famous sheep breed of Pakistan is Thalli, which belongs to Thall area of the Punjab. Economically importance traits in Thalli sheep include birth weight, weaning weight, yearling weight and greasy fleece weight (Hussain et al., 2006). Kajli is a thin tailed sheep which is found in the Sargodha and Gujrat districts of Punjab, Pakistan. This breed of sheep is well known for its good quality mutton and males are especially reared for sale as sacrificial animals and have well defined type traits (Qureshi, 2010). The most documented gene for selection of quantity and quality traits as double muscle and meat tenderness is calpastatin gene, which is located at 5q15 region on fifth chromosome of sheep (Gabor et al., 2009). Genetic variation in *CAST* gene and the effect on meat quality traits both in cattle and sheep has been reported by many researchers (Casas et al., 2006; Schenkel et al., 2006; Palmer et al., 2000; Zhou et al., 2007). Calpastatin

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Abbreviations: CAST, Calpastatin; **PCR,** polymerase chain reaction; **RFLPs,** Restriction fragment length polymorphisms.

encoded by *CAST* gene inhibit the calpains system. The calpain-calpastatin system comprises a family of calcium dependent neutral proteinases, with *CAST* acting as a specific inhibitor of µ and m-calpain proteases (Merin et al., 1998).

At the molecular level, calpastatin protein is composed of five-domains having molecular weight of 76 kDa which bind and inhibit the calpains system. The function of N terminal domain is to enhance the targeting of the inhibitory region of calpain system by other inhibitory domain but itself don't play any inhibitory role (Averna et al., 2001).

The remaining four inhibitory domains consist of three highly conserved regions namely: A, B and C which have the capability to inhibit the calpains independently (Cong et al., 1995). The two regions A and C binds to calpain in Ca^{2+}-dependent manner but don't inactivate the system itself, while B region inhibits calpain (Mohammadi et al., 2008; Amanda et al., 2006).The physiological and genetic role of calpastatin gene in meat tenderness has been reported in various animals (HuffLonergan et al., 1995; Boehm et al., 1998). The levels of calpastatin gene expression differ among species, breeds and muscles (Geesink and Koohmaraie, 1999). To study meat quality in sheep by using a molecular genetic approach, Palmer et al. (1999) have chosen the ovine *CAST* gene as a candidate gene for meat quality.

Marker assisted selection is one of the new DNA based methods that improves accuracy and progress in animal selection programmes (Bastos et al., 2001). Genetic markers are important for the determination of allelic polymorphism at any specific locus. A two-allele system of polymorphic variants (M and N) of the ovine *CAST* gene by a PCR-RFLP method has been described (Palmer et al., 1998). Also a three-allele system for the ovine and cattle *CAST* genes by PCR-SSCP has been reported (Palmer et al., 1998). Genetic markers have also important role in marker assisted selection and mapping quantitative trait loci (QTL) (Hiendleder et al., 2003; Kuhn et al., 2003). The existence of genetic polymorphisms associated with productive traits allows the breeder to develop desire traits in specific animals (Erhardt et al., 2007). PCR-RFLP is one of the most commonly used methods for polymorphism genotyping due to its simplicity. Initially, the RFLP analysis required a radio-actively labeled probe for detection, and now the method is coupled with PCR and simple agarose gel electro-phoresis (Cohen et al., 2002). The objectives of the present study are as follows:

(i) To investigate the genotypic and allele frequencies of a CAST gene polymorphism in Kajli, Lohi and Thalli breeds.
(ii) Investigation about the Hardy-Weinberg equilibrium of CAST gene polymorphism in population of Kajli, Lohi and Thalli sheep breeds.

MATERIALS AND METHODS

Blood sample collection

Random blood sample were collected from 300 Pakistani sheep (100 samples from each Thalli, Lohi and Kajli breeds). These samples were collected from different government as well as private farms in Pakistan.

Lohi breed animals were provided by livestock production and research institute, Bahadur Nagar, Okara, while Thalli breed animals were provided by sheep and goat research farm layya multan and Kajli breed blood samples were provided by National Agriculture Research Center, Islamabad. All the animals were purebred. Approximately 5 ml of blood were collected from each animal in vacutainer containing EDTA and stored at 4°C. The blood samples collected were used for genomic DNA isolation.

Genomic DNA extraction and dilution

Genomic DNA was extracted from whole blood using phenol chloroform extraction method (Sambrook et al., 1989). The confirmation of extracted DNA was carried out on 1.5% agarose gel. The extracted DNA was diluted in the ratio of 1/50 in distilled water and used in polymerase chain reaction (PCR) amplification.

PCR amplification

Veriti 96-well thermal cycler (Applied Biosystems, CA) was used for PCR using Taq polymerase (MBI Fermentas). PCR was carried out in a total volume of 25 µl which consisted of 2.5 µl of template genomic DNA, 2.5 µl 10× $(NH_4)_2SO_4$ PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 1 µM each of forward and reverse primers and 2.5 U of recombinant *Taq* DNA polymerase (MBI Fermentas).

Primers, described in a previous study (Palmer et al., 1998), located on exon 1C/1D region of the ovine *CAST* gene. These primers were used in PCR to amplified partial regions (622 bp) of exon 1C and 1D and the intron between them. The primers' sequences are: forward primer (exon 1C): 5' TGGGGCCCAAT-GACGCCATCGATG 3' and reverse primer (exon 1D): 5' GGTGGAGCAGCACTTCTGATCACC 3'. The PCR reaction was carried out with an initial denaturation temperature of 95°C (5 min); cycling reaction (35 cycles) of 95°C (60 s), 62°C (60 s) and 72°C (120 s), final extension at 72°C for 8 min.

Restriction fragment length polymorphism (RFLP) analysis

The resulted amplicon of 622 bp was digested with four base cutter *Msp1* restriction enzyme. The digestion reaction was carried out in 20 µl of mixture which consists of 0.5 µl of *Msp1* restriction enzyme and 15 µl of PCR product. The reaction mixture was then incubated at room temperature for 12 to 16 h, after incubation the restriction fragments were visualized on 2.5% agarose gel containing ethidium bromide. Size of different DNA bands resulting from restriction with enzyme was confirmed through standard DNA marker and the size of unrestricted PCR product was taken as the control.

Statistical analysis

The allelic and genotypic frequencies, observed heterozygosity (H_O) and expected heterozygosity (H_E) and Chi-square (χ^2) values for the Hardy-Weinberg equilibrium were calculated as described (Rosner, 2005).

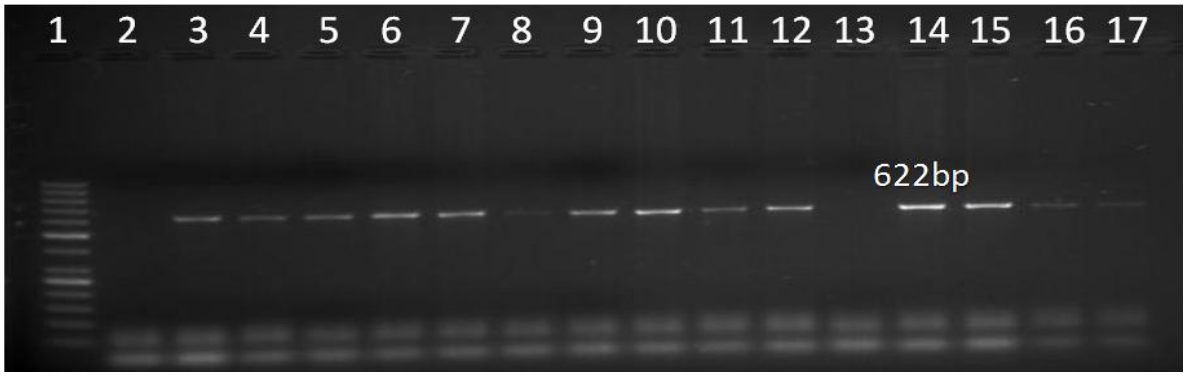

Figure 1. Analysis of amplified products of *CAST* gene (Exon 1C and 1D) on 2% agarose gel. Lane 1: 50 bp DNA ladder (MBI, Fermentas); Lane 2: Negative control; Lane 3-7: Amplified Product (622 bp) of Lohi *CAST* gene; Lane 8-12: Amplified product (622 bp) of Thalli *CAST* gene; Lane 13-17: Amplified product (622 bp) of Kajli *CAST* gene.

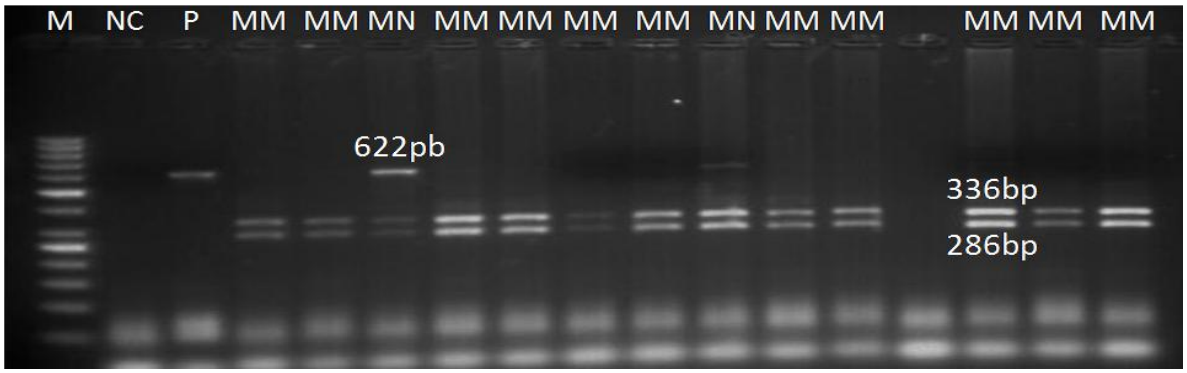

Figure 2. Restriction analysis of amplified product of Thalli *CAST* gene (Exon IC/ID) with Msp1 on 2.5% agarose gel electrophoresis. Lane M: 50 bp DNA ladder (MBI, Fermentas); Lane NC: Negative control; Lane P: PCR product of thalli *CAST* gene (622 bp); Lane MM: Homozygous genotype (336 and 286 bp) of Thalli sheep breed; Lane MN: Heterozygous genotype (622, 336 and 286 bp) of Thalli sheep breed.

RESULTS

Analysis of PCR amplified CAST gene fragment

The analysis of Calpastatin (*CAST*) gene polymorphism was carried out using PCR-RFLP. The exon 1C/1D and introns between them of the ovine *CAST* gene locus was amplified. The amplified product shows the amplification of 622 bps DNA fragment in all the analyzed samples of Lohi, Kajli and Thalli animals. The Figure 1 shows the amplified products of *CAST* gene in Lohi, Kajli and Thalli animals.

Digestion of PCR product with *Msp*1 restriction enzyme

The digestion of 622 bp PCR product amplified from exon 1C/1D region of *CAST* gene differentiated alleles M and N. The *Msp*I digestion fragments were of 336 and 286 bp for allele M (having restriction site for *Msp*I) and of 622 bp for allele N. Thus, the PCR-RFLP profile from animals homozygous for the M allele (MM) produced two bands of 336 and 286 bp. Three bands of sizes 622, 336 and 286 bp were seen in case of heterozygous genotype (MN), while the PCR-RFLP from animals homozygous for the N allele (NN) showed a 622 bp band only as shown in Figure 3.

RFLP analysis of Thalli, Lohi and Kajli sheep breeds.

In population of Thalli sheep, only two genotypes (MM and MN) were detected. The homozygous genotype MM and heterozygous genotype MN was detected in 80 and 20 sheep animals respectively. The homozygous genotype NN was not found in this population of sheep (Figure 2). The allelic frequencies for M and N in this breed were 90 and 10% (Table 1).

In the population of Lohi sheep breed, the frequencies of MM and MN genotype was 77 and 20% respectively, while the frequency of NN genotype was 3% as shown in

Table 1. Genotypic and allelic frequencies of the *Msp1* polymorphism in *CAST* gene of Thalli, Lohi and Kajli Sheep Breeds.

Breed	N	Genotypic frequency			Allele frequency	
		MM	MN	NN	M	N
THALLI	100	80	2	0	0.9	0.1
LOHI	100	77	20	3	0.87	0.13
KAJLI	100	68	26	6	0.81	0.19

N = Total number of animals.

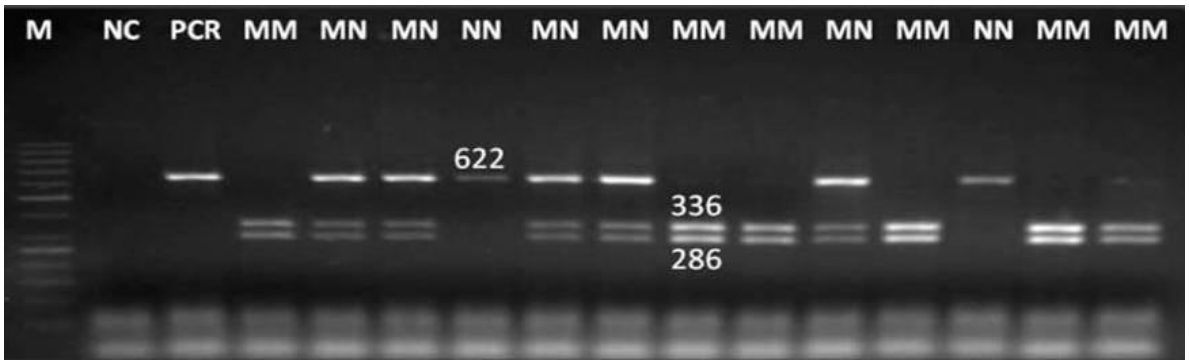

Figure 3. Restriction analysis of amplified product of Lohi and Kajli *CAST* gene (Exon IC/ID) with *Msp*1 on 2.5% agarose gel electrophoresis.Lane M: 50 bp DNA ladder (MBI, Fermentas); Lane NC: Negative control; Lane PCR: PCR product of *CAST* gene (622); Lane MM: Homozygous genotype (336 bp and286 bp) of Lohi and Kajli sheep; Lane MN: Heterozygous genotype (622 bp, 336 bp and 286 bp) of Lohi and Kajli sheep breed.

Table 2. Observed, expected heterozygosity and chi- square values for the Hardy-Weinberg equilibrium.

Breed	N	H_O	H_E	χ^2
THALLI	100	0.10	0.18	1.23^{NS}
LOHI	100	0.10	0.226	1.336^{NS}
KAJLI	100	0.10	0.31	2.39^{NS}

H_O = Observed Heterozygosity; H_E = Expected Heterozygosity; χ^2= Chi-square; NS = Non-significant (*p<0.05*).

Table 1. The calculated frequencies for M and N were 82 and 18% respectively. The representative bands for MM, MN and NN genotypes for Lohi sheep breeds are shown in Figure 3. Three genotypes were detected in Kajli animals (Figure 3). The calculated allelic frequencies were 81 and 19% for M and N respectively. The calculated genotypic and allelic frequencies of Kajli breed are shown in Table 1.

Hardy-Weinberg equilibrium

The H_o (observed) and H_e (expected) heterozygosity for Thalli sheep breed were 0.10 and 0.18 respectively, while the Chi-Square value for this breed was 1.23. For Lohi sheep breed, the H_o, H_e and χ^2 values were 0.10, 0.226 and 1.336 respectively. The calculated values for H_o, H_e and χ^2 in Kajli breed were 0.13, 0.31 and 2.39. At one DF (P< 0.05) the calculated χ^2 values were less than the tabulated values, so all the χ^2 values for three studied breed were non significant. The chi-square test showed that the three animal populations used in this study were in HWE (Hardy-Weinberg equilibrium) for the analysed CAST locus. H_O and H_E values and the chi-square value for the Hardy-Weinberg Equilibrium are shown in Table 2.

DISCUSSION

Detection of polymorphism which may belong to meat growth could prolific to the beef industry. Calpastatin is the most documented gene for selection of quantity and

quality traits as double muscle and meat tenderness in animals (Gabor et al., 2009). The frequency of M allele was higher than the allele N frequency in the three studied sheep population. Our genotypic and allelic frequency for three sheep breeds were in agreement with those of sharoudi et al. (2006) who observed frequencies of 61, 36 and 3% of the MM, MN, and NN genotypes respectively in Iranian karakul sheep. In Iranian kurdi sheep, the frequencies of 76, 24 and 0% were observed for MM, MN and NN genotype respectively, showing the existence of M allele more frequent then N allele (Nassiry et al., 2007). Similar data were found by Mohammadi et al. (2008) who studied polymorphism of *CAST* gene in 111 Arabic sheep breed and observed the frequencies of 70.27, 28.28 and 0.9% of the AA, AB and BB genotypes (Corresponding to MM, MN and NN genotypes) respectively. In another report Gabor et al. (2009) investtigated the random samples of 96 sheep breeds of Lacaune, Lacaune x Tsigai, Tsigai, Valachian and East Friesian and observed the genotypic frequency of 87% for MM, 13 and 0% frequencies for MN and NN genotypes respectively. The allelic frequencies were observed to be 77% for the M and 23% for the N allele in 22 distinct Corriedale rams (Palmer et al., 1998). The present data was also similar to Lan et al. (2009), who found the frequency of 95.9% for allele T and 4.1% for allele C (corresponding to allele M and N). The frequency of 69 and 31% for allele C and G (corresponding to allele M and N) were obtained by Kubiak et al. (2004) illustrated that allele C was more frequent then allele G. Our results were also supported by other researchers (Gorbani et al., 2009; Schenkel et al., 2006; Chitra and Aravndakshan, 2004).

In contrast to these results, a frequency of 50% of M allele was detected in Ghezel x Arkharomerino sheep. A high frequency of genotype MN in Arkharomerino (47.62%) and Ghezel x Arkharomerino (46.67%) sheep was observed. The NN genotype was not found in these animals (Elyasi et al., 2005). In recent study, Majidi et al. (2009) observed the frequency of 42% for allele A and 58% for allele B (corresponding to allele M and N) in 41 nelor cattle.

Kurdi sheep was genotyped using single-strand conformation polymorphism PCR (PCR-SSCP). Three alleles a, b, c and three genotypes aa, ab, ac were detected in the sheep genotypes tested. The allele with the highest frequency recorded was allele a (78%) and the most common genotype was aa having frequency of 54.76% (Nassiry et al., 2006). Similarly, Tahmoorespour et al. (2005) found frequency of 70% for allele a, that of 8% for allele b and 22% for allele c in Baluchi and Kurdi sheep respectively. The frequencies of 69 and 70% for allele a were observed in Dorset Down and Coopworth sheep breeds respectively (Palmer et al., 1999).

Several new single nucleotide polymorphisms (SNPs) have recently been reported in ovine, bovine and caprine *CAST* genes by PCR-RFLP, PCR-SSCP and DNA sequencing (Byun et al., 2008; Zhou et al., 2007; Zhou and Hickford, 2008). Small ruminants calpastatin gene mRNA transcript variants 2 and 4 have also been reported (Zhang et al., 2009).

Further studies on calpastatin gene in Pakistani sheep should be carried out to investigate the recently reported *CAST* gene polymorphism, to detect novel polymorphisms and to test the association of such polymorphisms with growth and meat quality traits in these animals.

This was the first study on *CAST* gene polymorphism in Pakistani Thalli, Lohi and Kajli sheep animals and it represents the first step in the investigation of DNA polymorphism affecting growth rate in these breeds.

Conclusion

On the basis of results obtained, it can be concluded that the allelic and genotypic frequencies of calpastatin gene locus investigated in this study appear to be similar in different sheep breeds. The genotype MM and allele M was present in high frequencies in the studied Thalli, Lohi and Kajli sheep. The χ^2 test confirmed that the populations were in HWE (Hardy-Weinberg equilibrium) for the tested locus.

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