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Calpastatin (*CAST*) Gene Polymorphism and its Association with Average Daily Weight Gain in Balkhi and Kajli Sheep and Beetal Goat Breeds

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Abstract.- Calpastatin (*CAST*) gene polymorphism has been shown to affect growth rate in sheep. *CAST* gene polymorphism using a PCR-RFLP and its effect on live weight gain in Balkhi and Kajli sheep and Beetal goat breeds were investigated. Alleles M and N of the *CAST* gene were detected by digestion of a 622 bp PCR product with the *MspI* restriction enzyme. Frequencies of MM, MN and NN genotypes were found to be 76%, 24% and 0% in Balkhi breed and 74%, 24% and 2% in Kajli breed, respectively. Only the MM genotype was detected in Beetal goats. In both the sheep breeds the MN genotype animals showed significantly higher weight gain as compared to the other genotypes. Effect of the genotype on average daily weight gain was observed to occur from birth to eight months age in the Balkhi breed and from birth to four months age in the Kajli breed. This showed that the A>G polymorphism in the exon 1C/1D region of the ovine *CAST* gene, investigated in this study is a potential molecular marker for marker assisted selection (MAS) concerning growth rate in both Balkhi and Kajli sheep breeds.

Key words: Calpastatin, genotypes, growth rate, PCR-RFLP.

INTRODUCTION

In Pakistan sheep breeds are raised for meat and carpet wool purposes (Akhtar, 1993). To cater for the quickly growing human population in Pakistan more food supply is required. Increase in sheep production will help increase mutton production and MAS can play a very important part to better sheep production in Pakistan. Research efforts into QTLs are under way internationally and a number of commercial DNA tests have already become available for some QTLs in sheep (Van der Werf, 2007).

Rate of synthesis and degradation of muscle proteins affects rate and extent of skeletal muscle growth. Calpastatin encoded by the *CAST* gene is a part of the calpain system. Calpain system comprises three molecules: two calcium dependent proteases, μ-calpain and m-calpain, and a third polypeptide, calpastatin, whose function is to inhibit the two calpains (Goll *et al*., 2003). Calpain system is important in skeletal muscle formation and degradation and in meat tenderness after slaughter. Decrease in activity of calpain system, due principally to a large increase in calpastatin activity results in decreased muscle protein degradation, which can result in increased rate of skeletal muscle growth (Goll *et al*., 1992).

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, 2000). Also Palmer *et al*. (1997), Tahmoorespour *et al*. (2005) and Chung *et al*. (1999) have described a three-allele system for the ovine and cattle *CAST* genes by PCR-SSCP. Previous studies have shown an association of the *CAST* gene polymorphism with increased live weight gain in sheep (Palmer *et al*., 1999, Tahmoorespour *et al*., 2005; Nassiry *et al*., 2006) and *CAST* has been suggested as a candidate gene for MAS in sheep (Palmer *et al*., 1997).

There are 28 sheep and 34 goat breeds in Pakistan (Isani and Baloch, 1996). Balkhi and Kajli are two of the fastest growing sheep and Beetal is one of the fastest growing goat breed of Pakistan. Balkhi is found in the Khyber Puktunkhwa and Kajli and Beetal breeds are found in the Punjab province of Pakistan (Khan, 2006). The purpose of the

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present study was to analyze *CAST* gene polymorphism and to study its association with average live weight gain in the Balkhi, Kajli and Beetal breeds. This is the first report which shows the association of an A>G polymorphism in exon 1C/1D region of the *CAST* gene with average daily weight gain in two Pakistani sheep breeds.

MATERIALS AND METHODS

Animals

Three hundred animals each of Balkhi, Kajli and Beetal breeds kept at Livestock Research Station of the National Coordinated Small Ruminants Research Program, Animal Sciences Institute, National Agricultural Research Centre, Islamabad, the Government Livestock Experimental Station, Khoshab and at private farms in Punjab and Khyber Pukhtunkwa Provinces were used in this study. The animals were selected randomly at the farms where the study was conducted. All the animals were purebred. Fifty rams of each sheep breed and 50 bucks of the goat breed had been kept at these farms for breeding purpose and 900 animals used in this study had been sired by these animals. The dams that gave birth to these animals were also almost of the same age. For measuring weight gain, weights of animals at birth and then at four, eight and twelve months of age were recorded. In order to exclude the effect of sex on weight gain only male animals were used in this study. The weaning time for all the animals was four months. All the animals used in this study were born normally, as singlets and were kept on the same food and under the same management conditions.

Genomic DNA extraction

Blood samples from all animals were collected in EDTA-coated vacutainers (EC, EDTA.K3). Genomic DNA was extracted from blood using Genomic DNA Purification Kit (Promega, Cat. No. A1120) following manufacturer's protocol.

Polymerase chain reaction (PCR)

Primers, described in a previous study (Palmer *et al*., 1998), located on exon 1C/1D region of the ovine *CAST* gene, were used in the PCR. The reaction amplified partial regions of exon 1C and 1D and the intron between them.

Forward primer (exon 1C):
5' TGGGGCCCAATGACGCCATCGATG '3
Reverse primer (exon 1D):
5' GGTGGAGCAGCACTTCTGATCACC '3

PCR was carried out in a total volume of 50 µl which consisted of 50 ng of template genomic DNA, 5 µl 10X $(NH4)_2SO4$ 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, Cat No. EP0401). The PCR was carried out in Veriti 96-well Thermal Cycler (Cat No. 4375786, Applied Biosystems, CA). The cycling conditions consisted of an initial denaturation at 95°C (5 min), followed by thirty five cycles at 95°C (1 min), 62°C (1 min), 72°C (2 min), which was followed by final extension at 72°C (8 min).

Restriction fragment length polymorphism (RFLP)

PCR product (20 µl) was mixed with 5 U of the restriction enzyme *Msp*I (MBI, Fermentas, Cat No. ER0541), 3 µl of Tango buffer and 7 µl H_2O and incubated overnight at 37°C. The restriction digest products were electrophoresed on 2% agarose gel in 1X TAE buffer and PCR-RFLP bands were visualized under UV light by ethidium bromide staining.

Statistical analyses

The allelic and genotypic frequencies, observed heterozygosity (H_O) and expected heterozygosity (H_E) values and chi-square (χ^2) values for the Hardy-Weinberg equilibrium were calculated as described (Rosner, 2005). Average daily weight gain (in grams) from birth to weaning (WG4), weaning to eight months of age (WG8) and eight to twelve months of age (WG12) were calculated by subtracting the final weights from the initial weights and dividing by the number of days. General linear model (GLM) was used to study the effect of genotype on average daily weight gain. GLM was performed using the MiniTab software (Mintab Inc, PA, USA). The effect of the genotype was taken as a fixed effect and the sire effect was

included as a random effect. Least square means (LSM) were compared by least significant difference (LSD), which was calculated using SPSS software v 13 (SPSS, Chicago, IL).

RESULTS

PCR-RFLP

The PCR resulted in an amplicon of 622 bp in case of all the genomic DNA samples. Restriction digest of this fragment with the restriction enzyme *MspI* detected an A to G substitution in the intron between exons 1C and 1D (GenBank accession number AF016006, AF016007 and AF016008). The restriction digest produced 336 and 286 bp bands in case of the allele having the *MspI* restriction site in it (designated allele M). An uncut 622 bp band was seen for the allele lacking the restriction site in it (designated allele N). Thus the PCR-RFLP 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 (Fig. 1).

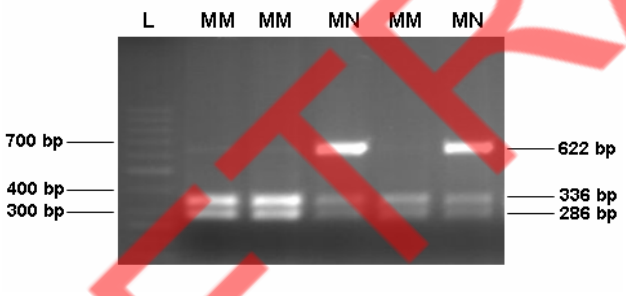

Fig. 1. PCR-RFLP from five animals. L, DNA size marker 50 bp DNA ladder; MM, genotype MM (336 and 286 bp bands); MN, genotype MN (622, 336 and 286 bp bands).

Genetic variability

The observed percentage of genotypes and allelic frequencies, H_O and H_E values and the χ^2 value for the Hardy-Weinberg Equilibrium are shown in Table I. The χ^2 test showed that the three animal populations used in this study were in Hardy-Weinberg equilibrium ($P < 0.05$). All the animals of Beetal breed were found to have the MM genotype.

Association analysis

GLM analysis showed that the effect of *CAST* genotype was highly significant on the WG4 ($P = 0.00$) and the WG8 ($P = 0.00$) traits in case of the Balkhi breed. There was no significant effect on the WG12 trait ($P = 0.77$). In case of Kajli breed the effect of genotype was also highly significant but only on the WG4 trait ($P = 0.00$). LSM and standard errors (SE) for each *CAST* genotype are shown in Table II. In case of the Balkhi breed comparison of LSM values of the two genotypes was done by subtracting one LSM value from the other. The results show that the MN genotype Balkhi animals gain more weight than the MM genotype animals. This weight gain is 37.8 g/day more from birth to four months age and 16.2 g/day more from four to eight months age in MN genotype animals. Comparison of the LSM values was done by LSD in case of Kajli animals. Results show that Kajli animals of the MN genotype grow faster by 13.2 g/day ($P = 0.00$) compared to MM genotype Kajli animals and by 44.5 g/day ($P = 0.00$) compared to MM genotype Kajli animals from birth to four months age. The MM genotype Kajli animals gain 31.2 g/day ($P = 0.02$) more weight than Kajli animals of NN genotype from birth to four months age.

DISCUSSION

In our study the frequency of the MM genotype in Balkhi and Kajli sheep was found to be 76% and 74%, respectively. The observed frequency of the MN genotype was 24% in both the sheep breeds and the frequency of NN genotype was 0% and 2% in Balkhi and Kajli breeds, respectively. We also attempted to search for any relationship between the genotype and weight gain in Beetal goats. But we found no polymorphism in the goat breed and only the MM genotype was detected in this breed. The Beetal data presented in Table II is just to show average weight gain in this breed.

Our genotype and allele frequency results for Balkhi sheep are in agreement with those of Nassiry *et al*. (2007) who observed frequencies of 76%, 24% and 0% for the MM, MN and NN genotypes respectively in Iranian Kurdi sheep. Sharoudi *et al*. (2006) reported frequencies of 61%, 36% and 3%

Table I.- Genotypic and allelic frequencies, observed and expected heterozygosity and chi-square value for the Hardy-Weinberg equilibrium.

Breed	n	Genotypes (percentage)			Allelic frequency		H_O	H_E	χ^2
		MM	MN	NN	M	N			
Balkhi	300	76	24	0	0.88	0.12	0.24	0.21	2.22^{ns}
Kajli	300	74	24	2	0.86	0.14	0.24	0.24	0.00^{ns}
Beetal	300	100	0	0	1.00	0.00	0.00	0.00	0.00^{ns}

H_O, Observed heterozygosity; H_E, Expected heterozygosity; χ^2,Chi-square value; ns, Non-Significant.

Table II.- Least square means and standard errors for the WG4, WG8 and the WG12 traits by the breeds and genotypes.

Breed	Genotype	WG4 (gm/day) LSM±SE	WG8 (gm/day) LSM±SE	WG12 (gm/day) LSM±SE
Balkhi	MM	155.8^{a}±3.1	76.3^{a}±1.8	67.5±1.4
	MN	193.6^{b}±9.3	92.5^{b}±1.8	68.3±3.0
Kajli	MM	42.3^{c}±3.0	36.9±1.7	124.7±2.2
	MN	55.6^{d}±2.3	31.2±2.3	129.8±4.4
	NN	11.1^{e}±1.7	41.6±0.0	113.8±7.6
Beetal	MM	233.2±2.5	136.6±4.2	161.6±2.6

LSM, Least square means; SE, standard error; WG4: weight gain from birth to 4 months; WG8, weight gain from 4 to 8 months; WG12, weight gain from 8 to 12 months.
[a,b] Comparison within a column was done by subtracting one LSM value from the other.
[c,d,e] LSM with different alphabetical superscripts were significantly different ($P < 0.05$) as found by LSD.

for the MM, MN and NN genotypes respectively in Iranian Karakul sheep. In 22 unrelated Corriedale rams allele frequencies were found to be 77% for the M and 23% for the N allele (Palmer *et al*., 1998). In a study on Arabic sheep, Mohammadi *et al*. (2008) reported frequencies of 70.27%, 28.28% and 0.9% for the AA, AB and BB (corresponding to MM, MN and NN) genotypes respectively. In a recent study (Gabor *et al*., 2009), which involved a mixed population of 96 sheep of Tsigai, Improved Valachian, East Friesian, Lacaune and Lacaune x Tsigai breeds, cumulative observed frequencies of 87%, 13% and 0% were found for the MM, MN and NN genotypes respectively. In contrast to these results, Elyasi-Zaringhabaee *et al*. (2005) reported a frequency of 50% of M allele in Ghezel x Arkharomerino sheep. They observed a high frequency of genotype MN in Arkharomerino (47.62%) and Ghezel x Arkharomerino (46.67%) sheep. The NN genotype was not detected.

In our weight gain study heteryozygous (MN genotype) animals showed significantly higher weight gain than the other two genotypes from birth to eight months of age in the Balkhi breed and from birth to four months of age in the Kajli breed. However, since there were only six Kajli animals of NN genotype in this study, it is suggested a new study be conducted by including a higher number of NN genotype animals in order to verify the effect of this genotype on weight gain in Kajli animals.

Nassiry *et al*. (2006) used single-strand conformation polymorphism PCR (PCR-SSCP) to study genotypes in Kurdi sheep. They found three alleles *a*, *b* and *c* and three genotypes *aa*, *ab* and *ac*. The allele with the highest observed frequency was allele *a* (78%) and *aa* was the most common genotype (54.76%). They also studied association of these genotypes with average daily weight gain from birth to weaning, six to nine months, and from nine months to yearling age. Only the genotype *ab* was shown to effect average daily weight gain and only from birth to weaning. Similarly Tahmoorespour *et al*. (2005) found frequency of 70% of allele *a*, 8% of allele *b* and 22% of allele *c* in Kurdi and Baluchi sheep and only the *ab* genotype was shown to have significant effect on

average daily weight gain from birth to weaning. Palmer *et al*. (1999) observed frequencies of 69% and 70% of allele *a* in Dorset Down and Coopworth sheep breeds respectively. In contrast to the results of Nassiry *et al*. (2006) and Tahmoorespour *et al*. (2005), they found an association of the genotype *ac* with increased live weight gain and increased age-corrected carcass weight.

Several new SNPs have recently been detected 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). Ovine CAST mRNA transcript variants 2 and 4 have been reported (Zhang *et al*., 2009).

The results of our study indicate the *CAST* gene locus, investigated in this study, is a potential molecular marker for MAS concerning growth rate in both Balkhi and Kajli sheep breeds.

Since the Beetal goat breed appears to be fixed for this *CAST* locus, it is suggested other parts of the caprine *CAST* gene be investigated for their association with growth rate in Beetal goats. It is further suggested *CAST* gene in Pakistani sheep and goat breeds be researched for recently reported polymorphism, for novel polymorphism and for association of its polymorphism with growth rate in these animals.

This was the first report which showed the association of the *CAST* gene SNP A>G in the exon 1C/1D region (Palmer *et al*., 1998, 2000) with average live weight gain in two Pakistani sheep breeds. It was also the first study on *CAST* gene polymorphism and its association with growth rate in Pakistani small ruminant breeds and is a part of the efforts to understand polymorphism of the DNA loci affecting growth rate in these breeds.

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