RESEARCH ARTICLE

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Genetic diversity of calpastatin gene and its association with some biochemical parameters in sheep

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ABSTRACT
The aim of the present study was to investigate the genetic diversity of calpastatin (CAST) gene and its possible effect on some blood biochemical parameters associated with meat quality in sheep. By using the PCR-RFLP method were genotyped 117 animals from two sheep breeds – Ascanian Merino (AM) and Northeast Bulgarian Merino (NEBM). Several blood biochemical parameters were studied: ALT (alanine aminotransferase), ALP (alkaline phosphatase), AST (aspartate aminotransferase), creatinine, LDH (lactate dehydrogenase), and urea. Polymorphism in the CAST gene was found in both studied breeds. The results showed statistically significant differences between levels of AST, LDH, and urea and different genotypes in the CAST gene in Northeast Bulgarian Merino and Ascanian Merino sheep breeds.

Key words: calpastatin (CAST), PCR-RFLP, genetic diversity, blood biochemical parameters

Introduction

Sheep breeding is an important component of the economy of rural areas in Bulgaria, especially in the semi-mountainous and mountainous regions of the country, and is the basis of sustainable agriculture.

CAST is a valuable molecular marker for performing selection for meat production in sheep (Gool et al., 2003; Byon et al., 2009; Gandolfi et al., 2011; Sato et al., 2011; Khan et al. 2012). Higher expression of the calpastatin gene shows a positive effect on the amount of meat in the carcass but it has an inverse correlation with meat tenderness in sheep after slaughter. For this reason, the genetic diversity of this gene is extremely valuable in maintaining selection programs to improve the average weight gain and meat quality of different sheep breeds (Sazili et al., 2005; Azari et al., 2012).

The Blood Metabolic Profile (BMP) is a set of diagnostic procedures based on the determination of a number of blood indicators (Van Saun, 2000). For the identification of BMP in individuals, the values of biochemical parameters are most commonly used (Herdt et al., 2000; Antunović et al., 2009).

Changes in biochemical parameters are an indicator of animal health status (Martin & Aitken, 2000; Hindson & Winter, 2002). The values of these indicators are a prerequisite for accurate assessment and interpretation of the final results, and they also could be breed-specific and related to the genotype (Kaneko et al., 1997, Bertoni, 1999, Push, 2002).

The relationship between some blood biochemical variations and the productive and reproductive characteristics of sheep has been investigated by many authors (Shaharbabak et al., 2009, Antunovic et al., 2015).

The aim of the present study was the investigation of the genetic diversity of the CAST gene and its association with some blood biochemical parameters associated with meat quality in sheep.

This is the first study in Bulgaria and one of the few worldwide, where it was investigated the association between genetic diversity of CAST gene and mean values of some biochemical parameters related to meat quality in sheep.

Materials and Methods

Animals – origin and place of rising

Animals of the two sheep breeds were included in the study – 31 Ascanian Merino ewes (AM) reared in Kabiuk State Company and 86 Northeast Bulgarian Merino ewes (NEBM) reared in the Agricultural Experimental Station of Targovishte.

Approximately 3 ml of blood were taken from *vena jugularis* from 117 animals by single-needle and closed-vacuum GD® system containing anticoagulant EDTA according to the method of Miller et al. (1988). All animal welfare requirements were met when blood samples were obtained. Blood samples were stored at -20 ° C.

PCR-RFLP procedure

Extraction of the genomic DNA from whole blood was performed with commercial kits for manual isolation of DNA according to the manufacturer's instructions (QIAamp DNA Blood Mini Kit (Qiagen).

Concentration and quality of the isolated genomic DNA were determined by Biodrop spectrophotometer at 260-280 nm and agarose electrophoresis in 1% agarose gel (Healthcare) prepared with distilled water and TBE 10x buffer (0.09 mM Tris-borate pH 8.3, 2 mM EDTA) (Thermo).

The PCR reactions were performed using PCR thermocycler QB-96 (Quanta Biotech) in the total volume of 10 µl containing 40 ng DNA template, 0.2 µl dd H2O, 20 pM of each primer, and 5 µl of ready-to-use Red Taq Polymerase Master Mix (VWR). The primer set was suggested by Palmer et al., (1998):

F: 5'- TGG GGC CCA ATG ACG CCA TCG ATG -3'

R: 5'- GGT GGA GCA GCA CTT CTG ATC ACC -3'

PCR-RFLP analysis was applied to identify the genotypes of the tested animals for the CAST gene. The digestion reaction was carried out in a 10 µl final volume, containing 6 µl PCR product and 10 U/µl *MspI* restriction enzyme (Bioneer, England). PCR products were incubated at 37ºC for 15 h (overnight) in a thermo-block. The fragment sizes were determined using GeneRuler™ Ladder, 50 bp (Fermentas) supplied with 1 ml 6xDNA Loading dye (Thermo) on 2 % agarose gel and then visualized with photodocumentary system Canon UVDI (Major Science), under UV-light, after staining with 10, 000 × GelRed ™ Nucleic Acid Stain (Biotium Inc, USA).

Biochemistry analysis

For biochemical testing from each animal was collected 3 ml blood from vena jugularis by a closed blood collection system GD®, in vacuum tubes with a gel activator for blood serum separation. Blood samples were stored at 4 °C. All biochemical parameters ALT, ALP, AST, creatinine, LDH, and urea) were examined by semi-automatic biochemical analyzer Screen master LIHD-113 (Hospital Diagnostic) Germany, with ready tests "Human", following the manufacturer's instructions.

Data analysis

The mean values of the biochemical parameters were compared by Student's t-test by statistical software MS Excel 2010 in order to establish the relationship between the genotype and the corresponding value of the indicator.

Results and Discussion

In the present study ALT, ALP, AST, creatinine, LDH, and urea were investigated to check the influence of the factor genotype of the CAST gene on their mean values.

Table 1. *Allele frequencies, genotype frequencies, coefficient of inbreeding of CAST gene.*

Breed	n	Allele number		Allele frequencies		Genotype			Heterozygosity		Fis	χ2
		Ao	Ae	M	N	MM	MN	NN	Ho	He		
AM	31	2.00	1.52	0.79	0.21	0.61	0.35	0.04	0.331	0.354	-0.069	0.600^{ns}
NEBM	86	2.00	1.35	0.85	0.15	0.72	0.26	0.02	0.255	0.255	0.000	0.004^{ns}

AM – Askanian Merino sheep breed; Northeast Bulgarian Merino sheep breed; Ao – observed number of alleles; Ae – expected number of allele; n – total number of tested animals; M – wild allele; N – mutant allele; MM – wild homozygous genotype; MN – heterozygous genotype; NN – mutant homozygous genotype; Ho – observed heterozygosity ; He – expected heterozygosity; Fis – coefficient of inbreeding; χ2 – chi square coefficient

Table 2. *Effect of CAST genotype on the studied biochemical parameters in the Ascanian breed (n = 31).*

Parameter/Genotype	MM	MN
Urea, mmol/l	15.1	12.1
Creatinine, mmol/l	88.4	91.5
ALT, U/L	17.5	18.1
AST, U/L	120.5	148.6
AP, U/L	68.7	76.7
LDH, U/L	473.3	563.8

MM – wild homozygous genotype; MN – heterozygous genotype; ALT – alanine aminotransferase; AST – aspartate aminotransferase;; AP – alkaline phosphatase; LDH – lactate dehydrogenase

Genetic diversity in the CAST gene was found in both tested breeds. In the Ascanian merino sheep breed, there were found two genotype – wild genotype *MM* and heterozygous genotype *MN*. In the Northeast Bulgarian merino sheep breed, there were detected all three possible genotypes for the CAST gene – *MM*, *MN*, and *NN* (Table 1).

In Ascanian sheep, the values of biochemical parameters ALT, AST, ALP, creatinine, and LDH were higher in the heterozygous genotype *MN* compared to the homozygous wild genotype *MM*, but the differences were not statistically significant (p> 0.05). A statistically significant difference was observed only in the values of AST, where p = 0.007 (Table 2 and Figure 1).

In the Northeast Bulgarian merino sheep breed, the homozygous mutant genotype *NN* was with the highest value in AST – 121.90, and a statistically significant difference was found there (p<0.02). For the other parameters, there was no significant difference between their average values (Table 3 and Figure 2).

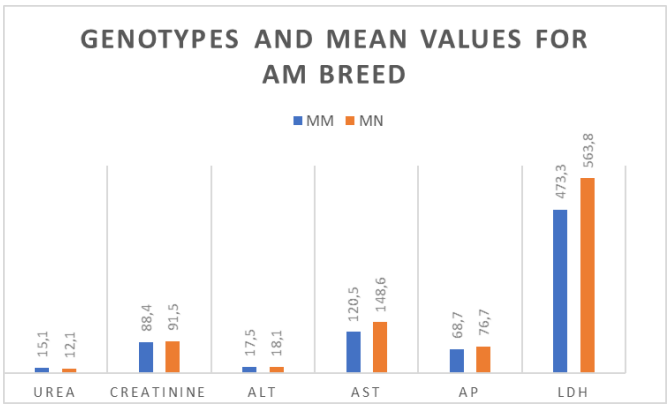

Figure 1. *Influence of CAST gene genotype on the average values of the biochemical parameters in Ascanian Merino animals.*

MM – wild homozygous genotype; MN – heterozygous genotype; ALT – alanine aminotransferase; AST – aspartate aminotransferase; AP – alkaline phosphatase; LDH – lactate dehydrogenase.

Table 3. *Effect of CAST genotype on the studied biochemical parameters in the Northeast Bulgarian Merino sheep breed (n=86).*

Parameter/Genotype	MM	MN	NN
Urea, mmol/l	7.50	7.47	6.80
Creatinine, mmol/l	69.06	69.20	52.00
ALT, U/L	22.87	21.20	18.70
AST, U/L	100.98	115.36	121.90
AP, U/L	176.24	211.32	199.20
LDH, U/L	557.62	541.04	718.55

MM – wild homozygous genotype; MN – heterozygous genotype; ALT – alanine aminotransferase; AST – aspartate aminotransferase; AP – alkaline phosphatase; LDH – lactate dehydrogenase.

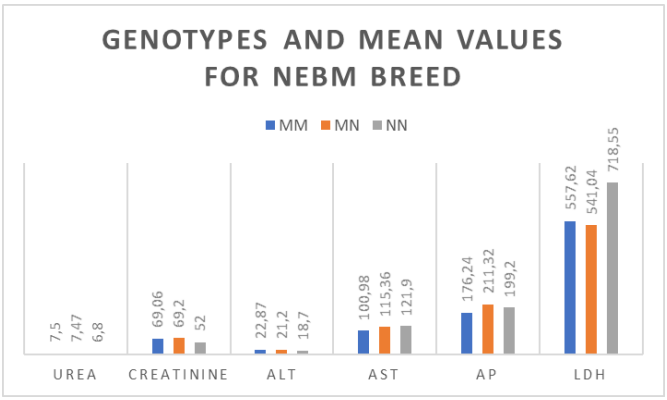

Figure 2. *Influence of CAST gene genotype on the average values of the biochemical parameters in Northeast Bulgarian Merino animals.*

MM – wild homozygous genotype; MN – heterozygous genotype; NN – mutant homozygous genotype; ALT – alanine aminotransferase; AST – aspartate aminotransferase;; AP – alkaline phosphatase; LDH – lactate dehydrogenase

In urea level, it was also observed statistically significant difference with a higher value for the homozygous genotype *MM* (p<0,01).

In the studied breed, it was found a statistically significant difference in the levels of LDH with a higher number in homozygous mutant genotype *NN* - 718.55 (p=0,02).

There is a lack of similar studies for the effect of the genotype of the CAST gene on biochemical parameters associated with meat quality in sheep. Jawasreh & Ismail, (2019), performed the only experiment close to ours. They studied the effects of calpastatin gene polymorphism on hematology and selected serum biochemical parameters in 31 Awassi lambs They detected two CAST genotypes with frequencies of 0.65 and 0.35 for *MN* (three major bands of 622, 336, and 268 bp) and *NN* (two major bands of 336 and 268 bp), respectively. Allele frequencies were 0.49 and 0.51 for *M* and *N* alleles, respectively. Animals with *MN MspI* CAST genotype had significantly (p < 0.05) higher neutrophil percentage and neutrophil to lymphocyte ratio but, significantly (p < 0.05) lower lymphocyte percentage and neutrophil to lymphocyte ratio than *NN MspI* CAST genotype. Serum T3 and cortisol concentrations were significantly (p < 0.05) higher in *MN MspI* CAST genotype than the *NN MspI* CAST genotype.

For the first time in sheep breeds grown in Bulgaria, it was studied the effect of the genotype of the CAST gene on

the values of biochemical parameters. Worldwide, the authors describe different studies of the effect of genotype and productive characteristics in sheep that are mainly related to the average daily gain and animal weight (Zapletal et al., 2010). In the available literature, we did not find other similar studies.

Conclusion

As a result, it can be concluded that there were statistically significant differences between different genotypes and obtained values in AST for the Ascanian breed and in AST, urea, and LDH in the Northeast Bulgarian Merino breed. The obtained results show that the CAST gene and the studied biochemical parameters can be used as a genetic marker in the selection for obtaining meat with better qualities. For the first time in Bulgarian sheep breeds, there have been studied the association of the CAST gene with some biochemical indicators, which are related to the health status and the quality of sheep meat. The present study could serve as a solid basis for more extensive research related to genotype and different parameters in sheep. When a definite association is established, the results could be implemented in practice and in preparation for breeding programs.

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