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# GENE AND GENOTYPE FREQUENCIES OF C3 COMPLEMENT COMPONENT ALLELES IN SHEEP BREEDS REARED IN BULGARIA

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**Summary**

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In order to determine the allele and genotype frequencies for the polymorphism of the gene coding the C3 component of the complement system, 432 sheep from nine breeds of different productive types were studied. Tests were carried out with Mouton Charollais, Ile de France, Trakia Merino breeds, milk crosses [Stara Zagora×East Friesian and (Stara Zagora × East Friesian) × Pleven Blackhead], White Maritsa, Patch-faced Maritsa, Karnobat, Pleven Blackhead and Stara Zagora breeds, with 48 animals studied from each breed. The animals were reared at the Agricultural Institute – Stara Zagora, the Agricultural Institute – Karnobat and private farms. Seven alleles were discovered (*S*, *S1*, *S5*, *S7*, *S10*, *F*, and *F1*), forming nine genotypes (*SS*, *SS1*, *FS*, *FS5*, *FS7*, *FS10*, *FF*, *FF1*, *F1F1*). The S allele frequency was the highest in sheep from the Stara Zagora and Trakia Merino breeds. In all other breeds, most common was the *F* allele, with this tendency being most evident in the Mouton Charollais and Pleven Blackhead breeds. The very rare *S1* allele was found in three breeds with a frequency of 0.01 to 0.05. Another rare allele discovered over the course of his study was *F1*, with frequency varying between 0.2 and 0.13. The frequencies of the nine established genotypes (*SS*, *SS1*, *FS*, *FS5*, *FS7*, *FS10*, *FF*, *FF1*, *F1F1*) varied in each breed. The *SS* genotype was most commonly encountered in the Stara Zagora breed (0.42), the heterozygous *FS* genotype was most common in the Mouton Charollais breed and milk crosses (0.23), whereas the animals homozygous for the *F* allele were prevalent among the Pleven Blackhead breed (0.54). The heterozygous *FS5* genotype was also notable, having a frequency of 0.33 in the Karnobat breed.

**Key words**: C3 complement component, polymorphism, sheep

## INTRODUCTION

The various genes in the mammalian genome predetermine different phenotype expressions. Along with the genes coding for factors of little economic significance, a major part influence the animals' productivity and resistance to various diseases. Studying the genetic structure of animal populations is a mechanism, through which selection can improve productivity, and increase natural resistance to infectious agents. To this end, a number of protein- and DNA-level analyses have been developed. The polymorphism of the complement system's C3 component is extensively researched in humans. A connection between some C3 alleles and the susceptibility (respectively resistance) to certain diseases has been found (Dissing *et al.,* 1972; Brönnestam, 1973; Brönnestam & Cedergren, 1973; Sorensen &

Dissing, 1975; Jans & Sorensen, 1980; Nylander *et al.,* 1990). The polymorphism of this gene in sheep has hardly been examined (Sotirov *et al.,* 2005a), motivating us to set the goal of determining the gene and genotype frequency of the polymorphic C3 system in sheep.

## MATERIALS AND METHODS

The investigations were performed in the spring and the summer of 2010. The polymorphism of C3 complement component was determined in sheep and rams from Mouton Charollais, Ile de France, Trakia Merino breeds, milk crosses [Stara Zagora × East Friesian and (Stara Zagora × East Friesian) × Pleven Blackhead], White Maritsa, Patch-faced Maritsa, Karnobat, Pleven Blackhead and Stara Zagora breeds reared at the Agricultural Institute in Stara Zagora, the Agricultural Institute in Karnobat and private farms. The total number of samples was 432 – 48 samples from each breed.

Blood for analysis was sampled in 3-mL vacutainers from *v. jugularis*. The blood was allowed to clot for one hour at room temperature (25 °C) and the samples were centrifuged at 4000 rpm for 10 min.

The polymorphism of C3 complement component was determined according to method of Teisberg (1970). The following buffers were used: a) stock solutions for gels – 5,5-diethylbarbituric acid sodium salt (Diemal Na, Loba-Chemie, Austria) 0,0230 M; 5,5-diethylbarbituric acid (Reanal, Hungary) 0.0037 M; Calcium-L(+)-lactate (Fluka AG, Switzerland) 0.0009 M; pH=8.6; b) stock solution for tray buffer – 5,5-diethylbarbituric acid sodium salt (Diemal Na, Loba-Chemie, Austria) 0.061 M; 5,5-diethylbarbituric acid (Reanal, Hungary) 0.01 M; calcium L(+) lactate (Fluka AG, Switzerland) 0.0018 M; pH=8.6. The first buffer was used for preparation of agarose gel (1%) where serum samples were applied. After their absorption, the electrophoresis was carried out at 20 V/cm for about 2.5 h. It was stained for 1 min with Amido Black 10 B and destained for overnight.

The destaining solution was prepared by mixing ethanol, distilled water and glacial acetic acid at a ratio of 5:5:1 (for example, 1 L destaining solution contains 450 mL ethanol, 450 mL distilled water and 100 mL glacial acetic acid). Then, 2 tablespoons of active charcoal powder were added in order to allow the manyfold use of the destaining solution. For the staining solution, 10 g amido black 10B was added to 1 L destaining solution (without active charcoal), shaken well, left for overnight, and if necessary, filtered after a week.

The gene and genotype frequencies were calculated by the method of Nicholas (1995), since it was earlier established that the alleles coding for the C3 complement component in sheep are inherited codominantly (Sotirov *et al.,* 2005b). The allelic and genotype frequencies within and between investigated breeds were compared by 2×2 contingency tables with the $\chi^2$ test set at 95% confidence interval and critical probability of 0.05.

## RESULTS

In order to establish the population's genetic structure, the relative frequencies of the alleles and the genotypes formed by the alleles were determined (Table 1). The highest frequency of the *S* allele was found in the Stara Zagora breed – 0.47, while at the same time the breed exhibited a very high frequency of the *F* allele – 0.43. The *S* allele frequency in the Stara Zagora breed was significantly higher than

**Table 1.** Relative allele frequencies of the C3 component of the complement system in sheep breeds, reared in Bulgaria

| Sheep breed | Allele | | | | | | |
|---|---|---|---|---|---|---|---|
| | *S* | *S1* | *S5* | *S7* | *S10* | *F* | *F1* |
| Mouton Charollais | 0.11 | 0.00 | 0.08 | 0.03 | 0.02 | 0.69 | 0.07 |
| Ile de France | 0.35 | 0.00 | 0.07 | 0.03 | 0.00 | 0.55 | 0.00 |
| Trakia Merino | 0.41 | 0.00 | 0.07 | 0.05 | 0.00 | 0.39 | 0.08 |
| Milk crosses | 0.25 | 0.00 | 0.05 | 0.02 | 0.01 | 0.59 | 0.08 |
| White Maritsa | 0.30 | 0.00 | 0.07 | 0.02 | 0.00 | 0.55 | 0.06 |
| Patch-faced Maritsa | 0.33 | 0.01 | 0.03 | 0.04 | 0.02 | 0.44 | 0.13 |
| Karnobat | 0.16 | 0.05 | 0.17 | 0.05 | 0.02 | 0.51 | 0.04 |
| Pleven Blackhead | 0.27 | 0.00 | 0.02 | 0.00 | 0.02 | 0.67 | 0.02 |
| Stara Zagora | 0.47 | 0.01 | 0.02 | 0.02 | 0.05 | 0.43 | 0.00 |

in White Maritsa ($\chi^2$=5.629, P<0.0177), Pleven Blackhead ($\chi^2$=8.068, P<0.0045), milk crosses ($\chi^2$=11.021, P<0.0009), Karnobat ($\chi^2$=21.818, P<0.0001) and Mouton Charollais ($\chi^2$=29.143, P<0.0001).

The relative frequency of the *F* allele was the highest in the Mouton Charollais (0.69) and Pleven Blackhead breeds (0.67), with the frequency of this allele being markedly high in all other breeds. The *F* allele rate in Mouton Charollais was significantly higher than in Karnobat ($\chi^2$=5.528, P<0.0187), Patch-faced Maritsa ($\chi^2$=11.167, P<0.0008), Stara Zagora ($\chi^2$=12.132, P<0.0005) and Trakia Merino ($\chi^2$=16.397, P<0.0001). The allelic frequency of allele *F* in Mouton Charollais was significantly higher than the frequency of alleles *S* ($\chi^2$=63.506, P<0.0001), *S5* ($\chi^2$=71.809, P<0.0001), *S7* ($\chi^2$=87.529, P<0.0001) and *S10* ($\chi^2$=90.991, P<0.0001).

Over the course of the study, several very rare alleles were found – *S1*, *S5*, *S7*, *S10* and *F1*. The alleles *S5*, *S7*, *S10* and *F1*, despite being considered rare, were found in nearly every breed with frequencies between 0.02 and 0.17, whereas the *S1* allele was present in only three breeds – Patch-faced Maritsa (0.01), Karnobat (0.05) and Stara Zagora (0.01).

The *F1* allele was observed in most of the investigated breeds, except for the Ile de France and Stara Zagora breeds. Its frequency was significantly higher in Patch-faced Maritsa than in Karnobat ($\chi^2$=4.364, P<0.0367) and Pleven Blackhead ($\chi^2$=7.705, P<0.0055).

The *S5* allele was observed in all investigated breeds with frequencies between 0.02 (Pleven Blackhead and Stara Zagora) and 0.17 (Karnobat). Its frequency was statistically higher in the Karnobat breed than in Ile de France, Trakia Merino and White Maritsa breeds ($\chi^2$=4.001, P<0.0455), milk crosses ($\chi^2$= 6.470, P<0.0110), Patch-faced Maritsa ($\chi^2$=9.872, P<0.0017), Pleven Blackhead and Stara Zagora breeds ($\chi^2$=12.015, P<0.0005). The frequency of this allele in Karnobat breed was statistically higher than those of *S1* and *S7* alleles ($\chi^2$=6.470, P<0.0110), *S10* ($\chi^2$=12.015, P<0.0005) and *F1* ($\chi^2$=8.037, P<0.0046).

It was established that the seven alleles reviewed thus far formed the following nine genotypes – *SS*, *SS1*, *FS*, *FS5*, *FS7*, *FS10*, *FF*, *FF1* and *F1F1*. Each genotype's frequency in the studied sheep breeds is presented in Table 2. The breeds with the highest prevalence of the *SS* genotype were the Stara Zagora and the Trakia Merino with respective frequencies of 0.43 and 0.35. The *SS* genotype frequency in Stara Zagora breed was significantly higher compared to White Maritsa ($\chi^2$=3.859, P<0.0495), Pleven Blackhead ($\chi^2$=4.848, P<0.0277), milk crosses ($\chi^2$= 10.338, P<0.0013) and Karnobat breed ($\chi^2$=22.004, P<0.0001).

The homozygous genotype *FF* was most prevalent among the Pleven Blackhead sheep with a frequency of 0.54. The frequency of this genotype was significantly higher in Pleven Blackhead sheep compered to Stara Zagora ($\chi^2$=6.171, P<0.0130), Patch-faced Maritsa ($\chi^2$=7.298, P<0.0069), Trakia Merino ($\chi^2$=12.995, P<0.0003) and Karnobat ($\chi^2$=14.755, P<0.0001). Among the Pleven Blackhead sheep the frequency of this genotype was significantly higher than the *SS* ($\chi^2$=11.378, P<0.0007), *FS* ($\chi^2$=18.750, P<0.0001) and *FS5*, *FS10*, *FF1* genotypes ($\chi^2$=29.042, P<0.0001).

The heterozygous *FS* genotype was most commonly found among the milk crosses and the Mouton Charollais breed (0.23). Its frequency was significantly higher in the milk crosses and the Mouton Charollais breed compared to Stara Zagora ($\chi^2$=3.872, P<0.0491) and Patch-faced Maritsa ($\chi^2$=5.352, P<0.0207).

Genotype *FS5* was observed in all investigated breeds, its frequency was significantly higher in the Karnobat breed compared to Ile de France, Trakia Merino and White Maritsa ($\chi^2$=4.631, P<0.0314), milk crosses ($\chi^2$=7.375, P<0.0066), Patch-faced Maritsa ($\chi^2$=11.090, P<0.0009), Pleven Blackhead and Stara Zagora ($\chi^2$= 13.402, P<0.0003). Among the Karnobat breed this genotype frequency was significantly higher than the frequency of genotypes *SS* and *FF1* ($\chi^2$=16.083, P<0.0001), *FS10* and *FF1* ($\chi^2$=13.402, P<0.0003), *SS1* and *FS7* ($\chi^2$=7.375, P<0.0066).

**Table 2.** Relative genotype frequencies of the C3 component of the complement system in sheep breeds, reared in Bulgaria

| Sheep breed | Genotype | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *SS* | *SS1* | *FS* | *FS5* | *FS7* | *FS10* | *FF* | *FF1* | *F1F1* |
| Mouton Charollais | 0.00 | 0.00 | 0.23 | 0.17 | 0.06 | 0.04 | 0.38 | 0.10 | 0.02 |
| Ile de France | 0.25 | 0.00 | 0.19 | 0.15 | 0.06 | 0.00 | 0.35 | 0.00 | 0.00 |
| Trakia Merino | 0.35 | 0.00 | 0.10 | 0.16 | 0.10 | 0.00 | 0.19 | 0.04 | 0.06 |
| Milk crosses | 0.13 | 0.00 | 0.23 | 0.10 | 0.04 | 0.02 | 0.38 | 0.04 | 0.06 |
| White Maritsa | 0.23 | 0.00 | 0.15 | 0.15 | 0.03 | 0.00 | 0.38 | 0.00 | 0.06 |
| Patch-faced Maritsa | 0.29 | 0.02 | 0.07 | 0.06 | 0.08 | 0.05 | 0.27 | 0.08 | 0.08 |
| Karnobat | 0.02 | 0.10 | 0.17 | 0.33 | 0.10 | 0.04 | 0.17 | 0.04 | 0.03 |
| Pleven Blackhead | 0.21 | 0.00 | 0.13 | 0.04 | 0.00 | 0.04 | 0.54 | 0.04 | 0.00 |
| Stara Zagora | 0.43 | 0.02 | 0.08 | 0.04 | 0.04 | 0.10 | 0.29 | 0.00 | 0.00 |

The genotypes *FS7*, *FS10*, *FF1* and *F1F1* were found in almost all breeds with frequencies of 0.02 to 0.10. The *SS1* genotype, considered to be exceptionally rare, was found only in three of the examined breeds – Patch-faced Maritsa (0.02), Karnobat (0.10) and Stara Zagora (0.02). The *FS5* genotype, even at low frequencies (0.04–0.17) was found in all breeds and was the most common genotype in the Karnobat sheep (0.33).

## DISCUSSION

Sotirov *et al.* (2005b) examined the prevalence and frequency of the C3 alleles in four breeds of sheep – milk crosses, Ile de France, Mouton Charollais and Trakia Merino breed (TMB). Their results showed that the *S* allele was the most prevalent among the milk crosses, Ile de France and TMB, whereas the most frequent allele for the Charollais Mouton breed was *F*. Our results confirmed the predominance of the *S* allele in TMB and of the *F* allele in Mouton Charollais. On the other hand, changes in the genetic structure of the Ile de France breed and the milk crosses were found, as the most common established allele was *F*, against the expectations for the domination of *S*. This reflects on the genotype level, with the change being most profound in the milk crosses, among which the frequency of the *SS* genotype was reduced threefold – from 0.36 to 0.13. This change was probably due to the purposeful selection of the animals for productivity, which replaced breeders with one genotype by individuals with another.

Sotirov *et al.* (2004) studied the effect of the C3 genotype on the concentration of serum lysozyme in different sheep breeds. In their study, the authors found five genotypes – *SS*, *FF*, *FS*, *FS5* and *FS7*, the *SS* genotype exhibiting significantly higher serum lysozyme levels.

Among the first to study C3 polymorphism in sheep were Gahne & Amorena (1987), who discovered a blood plasma protein similar to C3. Their experiments were initially conducted with agarose gel electrophoresis, and consequently with immunoblotting. They found four primary alleles, three of which (*F*, *I* and *M*) were represented by one main band, while the fourth (*S*) – by 3 to 5 bands. Family analysis indicated that the alleles were inherited in an autosomal codominant way.

The polymorphism of C3 in other animals is poorly investigated. Some of the few research teams in this respect were Gorman *et al.* (1981), who reported that the *C3F* and *C3S* alleles in dogs were inherited in a autosomal codominant manner, yet no other alleles could be found apart from these two primary ones. Kay *et al.* (1986), studied the C3 component polymorphism in horses and found three alleles, *C31*, *C32* and *C33*, which, according to them, were inherited codominantly. Some years later, Bowling & Dileanis (1990), found a fourth allele as well, which had a high frequency only in the Przewalski wild horse and a very low frequency (0.01–0.04) in three other breeds.

As mentioned above, the polymorphism of the C3 complement component in humans was thoroughly studied. Among its first investigators were Wieme & Demeulenaere (1967), who were surprised to discover that in some individuals, the zone of the $\beta_2$C globulin was represented by two bands, while examining the polymorphism of serum proteins in humans. The authors performed agarose gel electrophoresis (1%), yet obtained the same results as with starch gel electrophoresis. The consequent family analysis showed that these bands were inherited as

an autosomal sign, yet the authors did not specify exactly how. Their first discoveries gave a boost to research in this scientific field.

During the next year, another team (Alper & Propp, 1968) discovered five alleles by using 1% agarose gel, which offered a higher resolution. The authors studied the influence of the different C3 genotypes on blood serum concentration of the C3 component, the overall activity of the complement and the adhesion capability of C3 molecules, yet no significant differences were found. As a result of their experiments, the authors concluded that the *F1*, *F* and *S* alleles were inherited in a autosomal codominant way, whereas the inheritance of the *S1* and *F0.8* alleles occurred in a way that remained unknown.

Teisberg (1970) performed his research among Norwegians and Laplandians, to find out that the frequencies of their genotypes were significantly different. His studies on Norwegians led him to the discovery that the frequency of the *C3S* allele was 0.7865, and that of the *C3F* allele – 0.2082. When comparing these two frequencies, a difference of 0.0053 can be noticed, which is caused by the existence of seven very rare alleles (*C3F0.5*; *C3F0.8*; *C3F1.1*; *C3S0.6*; *C3S0.8*; *C3S0.4* and *C3S0.9*). A total of nine alleles determined the formation of the 13 different genotypes. The author used the term genotypes because he found out that they are inherited codominantly.

Botto *et al.* (1990) discovered the molecular mechanism responsible for the polymorphism of the C3 component. They determined that the difference between *C3S* and *C3F* resulted from a point mutation (cytosine to guanine) in nucleotide 364 of exon 3 of the C3-coding gene. This, in turn, leads to replacement of the amino acid arginine (in *C3S*) with glycine (in *C3F*). By using PCR RFLP, an absolute correlation between genome polymorphism and the distribution of *C3S* and *C3F* was established among 50 normal subjects. The authors pointed out an increased frequency of the *C3F* allele in people suffering from partial lipodystrophy, IgA nephropathy and Indian childhood liver cirrhosis. *C3F* could probably be used as a genetic marker for determining the susceptibility (respectively resistance) towards certain diseases.

With two other experiments (Dissing *et al.,* 1972; Sorensen & Dissing, 1975), it was established that the frequency of the *C3F* allele was higher in people suffering from atherosclerosis. According to the authors, the risk of that illness was 1.87 higher in *C3F*-positive individuals compared to the *C3F*-negative ones.

From the review done thus far it is apparent that the polymorphism of the C3 component of the complement system has been researched to a great extent in humans. Unfortunately, this issue has hardly been worked on in animals. To our best knowledge, there is no evidence in the available literature to suggest a correlation between C3 polymorphism and resistance or susceptibility to certain diseases in animals.

As a result of this study, the allele and genotype frequencies of the polymorphic gene responsible for the synthesis of the C3 component of the complement system were established in the nine examined breeds of sheep.

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