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Original scientific paper

POLYMORPHISM OF K-CASEIN AND B-LACTOGLOBULINE IN SIMMENTAL CATTLE IN SERBIA

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The objective of this study was to determine the frequency of the κ-casein and β-lactoglobulin genotypes in the Simmental cattle in Serbia and compare it with the frequency according to the Hardy-Weinberg equilibrium law. Blood samples were taken from a total of 157 cows of the Simmental breed in Toplica and Rasina districts. Of the 157 cows included in this study, the AA κ-casein genotype was found in 53 cows, which makes a frequency of 33.80%, the AB genotype in 81 cows or 51.60% and the BB genotype in 23 cows or 14.60%. The allelic frequency A was 59.60%, while allele B had a frequency of 40.40%. In regard to the frequency of genotypes and β-lactoglobulin alleles for the total studied population of cows obtained for AA, AB and BB genotypes for β-lactoglobulin, was 33.10%, 49.70% and 17.20%, respectively, which means that 52 animals had genotype AA, 78 genotype AB and 27 genotype BB. The frequency of alleles A and B resulting from the incidence of genotypes was 58.00% for allele A and 42.00% for allele B. The specified frequencies for both protein fractions statistically differed significantly from the frequency according to the Hardy-Weinberg equilibrium law, which confirmed the absence of equilibrium in the examined population.

Key words: β-lactoglobulin, κ-casein, polymorphism, PCR-RFLP, Simmental breed

INTRODUCTION

It has been sixty years since ASCHAFFENBURG and DREWRY (1955) discovered the existence of polymorphism for β-lactoglobulin in cow milk. Today, the polymorphism of α-, β-, γ-, κ-casein, β-lactoglobulin and α-lactoalbumin is well known. Their action on the quantity and composition of milk, in recent years, has become the subject of numerous research. In addition to

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the knowledge of the frequency of certain alleles in the cow population, and with the fact that these genes are bound by inheritance, the most favourable genetic variants can also be used in the selection of bulls used for artificial insemination.

The polymorphism of β-lactoglobulin (β-Lg) and κ-casein (κ-CN) proteins is incorporated into modern bovine breeding programs, with objective to functionally improve the populations. As the main protein of milk whey, β-lactoglobulin is determined by the genome positioned on the 11th bovine chromosome. CAROLI *et al.* (2009), in addition to two dominant polymorphic forms (A and B), also indicate nine rare polymorphic variants (C, D, E, F, G, H, I, J, W). K-casein, as one of the four casein milk proteins, is determined by the gene positioned on the 6th bovine chromosome. CAROLI *et al.* indicate fourteen polymorphic forms of κ-casein (A, A1, B, B2, C, D, E, F1, F2, G1, G2, H, I, J) of which four polymorphic forms are dominant (A and B).

The variant A of κ-casein has threonine (ASS) and aspartic acid (GAT) amino acids at positions 136 and 148 (LIN *et al.*, 1992). For variant B, isoleucine (ATC) is replaced with alanine (GTC). These differences are the result of a gene mutation in κ-casein (PINDER *et al.*, 1991). Similarly, β-lactoglobulin is also found in a large number of genetic variants in which variants A and B are dominant. The variants differ in 2 amino acids in the peptide chain. Variant A has aspartic acid (GAT) and valine (GTG) at positions 64 and 118, while variant B has glycine (GGT) and alanine (GTC), as stated by RACHAGANI *et al.* (2006). Milk produced by cows with AA genotype contains more lactoglobulin, and less casein and fat compared to milk deriving from cows with BB genotype (VAN DER BERG *et al.*, 1992).

Genetic polymorphism of κ-casein and β-lactoglobulin in cows of different breeds in Croatia was studied by IVANKOVIĆ *et al.* (2011). By using the new analytical methods, they determined the share of dominant allelic polymorphic variants of β-lactoglobulin and κ-casein in 3 commercial and 3 autochthonous breeds of cattle: Holstein, Simmental, Brown cattle, Buša, Slavonian-Srem Podolac and Istrian cattle. The share of allelic B variants of β-lactoglobulin is dominant in all researched breeds of cattle (>52.9%). The allelic A variant of κ-casein is dominant in selected breeds of cattle (60.7-76.4%), while share B variant of κ-casein was significantly more represented in autochthonous breeds of cattle (48.2-84.1%).

LIE *et al.* (2010) have studied the frequency of alleles and genotypes in Romanian Simmental and Holstein-Friesian cattle in Romania. In Simmental breed of cattle, κ-casein had two types of alleles, A and B, with a frequency of 0.761 and 0.239, while in AA, AB and BB genotypes the frequency was 0.582, 0.359 and 0.059, respectively, indicating a relatively low incidence of alleles B when comparing with A allele.

Genetic polymorphism of milk protein fractions and their effect on the production performance of Simmental cows in Poland was studied by FELENCZAK *et al.* (2006). These authors state that a genotype BD was also identified in the β-lactoglobulin, having a frequency of 0.024. Other genotypes had the following frequency: AA 0.236, AB 0.488 and BB 0.252, and alleles: A 0.486, B 0.502, D 0.012. Three genotypes were found for κ-casein: AA, AB, BB, frequency 0.281, 0.498, and 0.221, respectively, while for alleles it was: A 0.530 and B 0.470.

By examining the genetic polymorphism of κ-casein and its impact on the production performance of the Simmental breed, the autochthonous breed Buša, and the crosses of the Simmental and Red Holstein in Serbia, ĐEDOVIĆ *et al.* (2015) have found the following results: the genotypic incidence of κ-casein for Simmental breed were: 42.8; 47.6 and 9.6% for AA, AB and BB genotype, for crosses: 75.0; 25.0 and 0.0%, and for Buša animals: 41.7; 50.0 and 8.3%, respectively. The incidence of alleles A and B, for the observed breeds estimated on the basis of

the genotype frequency, had values of 0.667 and 0.333 for the Simmental breed, for crosses 0.875 and 0.125, and 0.667 and 0.333 for indigenous Buša breed of cattle, respectively, while working with PCR-RFLP analysis of β -lactoglobulin of a small herd of Simmental cows in Serbia CARO PETROVIĆ *et al.* (2017) have found the incidence of only AA β-lactoglobulin genotype.

The study of the frequency of genotypes of protein fractions is extremely important because in most new studies a positive effect of the B allele variant of κ-casein on the share of casein and total protein in milk is observed (MAO *et al.*, 1992; IKONEN *et al*., 1999a; KUCHEROVA *et al*., 2006; MOLINA *et al.*, 2006a; COMIN *et al.*, 2008; SITKOWSKA *et al.*, 2008). The milk with the κ-casein of the genotype BB requires a shorter period of curding (LUNDEN *et al.*, 1997; KUBARSEPP *et al.*, 2005), has a higher yield of cheese with a higher share of proteins (IKONEN *et al.*, 1999b; BRAUNSCHWEIG *et al.*, 2000; KWAI-HANG *et al.*, 2002a; MICEIKIENE *et al.*, 2005; MOLINA *et al.*, 2006b).

IKONEN *et al.* (1999b) note that the κ-casein B allele is associated with preferred coagulation properties, while CZERNIAWSKA-PIĄTKOWSKA *et al.* (2004) state that the B allelic variant of κ-casein shortens the coagulation time by 10 to 30%. SULIMOVA *et al.* (2007) confirm the importance of determining the κ-casein gene in cattle breeding and significant economic effects.

Numerous studies have sought to establish the relationships between polymorphic allelic variants of β-lactoglobulin and κ-casein, and lactation characteristics and process characteristics of milk (ANTUNAC *et al.*, 1991). The studies have confirmed the positive effect of AA genotype of β-lactoglobulin on milk performance (MAYER *et al.*, 1990; JAKOB and PUHAN, 1992; VAN DER BERG *et al.*, 1992; HILL, 1993; IKONEN *et al.*, 1999b; CAROLI *et al.*, 2004), although there are studies favouring the AB genotype of β-lactoglobulin (TSIARAS *et al.*, 2005; KARIMI *et al.*, 2009).

Recent studies confirm the beneficial effect of the BB-genotype of β-lactoglobulin on the milk fat content (TSIARAS *et al.*, 2005; BALCAN *et al.*, 2007; KARIMI *et al.*, 2009), the content of casein in milk (BRAUNSCHWEIG *et al.*, 2000), total protein content (BALCAN *et al.*, 2007) and higher yield of cheese (LUNDEN *et al.*, 1997; STRZALKOWSKA *et al.*, 2002) which is of great importance for the processing industry.

MALETIĆ *et al.* (2016) state that the PCR-RFLP (Polymerase Chain Reaction - Restricting Fragment Length Polymorphism) has enabled rapid analysis of polymorphisms of practically unlimited number of gene, including those encoding κ-casein and β-lactoglobulin. The objective of this study was to evaluate the polymorphism of κ-casein and β-lactoglobulin genes, as well as the distribution of genotypes in the Simmental population of cows.

MATERIALS AND METHOD

DNA isolation

Blood samples for genetic testing were taken from 157 cows of the Simmental breed in BD Vacutainer® K2EDTA tubes in the amount of 6 ml from the tail vein (v. Caudalis), and subsequently stored at a temperature of 4ºC until the DNA was isolated. DNA isolation was carried out using UltraClean® BloodSpin® DNA Isolation Kit (MO BIO Laboratories Inc., USA), according to the manufacturer's instructions for isolating DNA from the blood. In short, in order to obtain isolated DNA, 100 µl of the proteinase K solution is added first in 200 µl of first blood. Subsequently, 200 µl of B1 solution, 200 µl of B2 solution, 500 µl of B3 solution, 500 µl of B4 solution and finally 100 -200 µl of B5 solution are added. After adding each of

these solutions, centrifugation of the content is performed, at different duration and speed. For the Polymerase chain reaction (PCR) in the 20 μl reaction, the following was required: sterile deionized water 13.4 μl, PCR buffer (1X) 2 μl, $MgCl_2$, dNTP (200 μM) 0.5 μl, 1 μl (0.4 μl μM) of each primer, Taq polymerase (0.02 U/μl; Kapa B 0.1 μl; Kapa Biosystems, USA) and 2 μl of isolated DNA each. The multiplication of the κ-casein gene portion containing the polymorphic sequence was done using the following primers (MITRA *et al.*, 1998): Kasein FW 5′ CAC GTC ACC CAC ACC CAC ATT TATC - 3′ and Kasein REV 5′ TAA TTA GCC CAT TTC GCC TTC TCT GT - 3′ (Invitrogen-Thermo Fisher Scientific Inc., USA). The multiplication of the β-lactoglobulin gene portion containing the polymorphic sequence was done using the following primers MEDRANO and AGUILAR-CORDOVA, 1990): β-lactoglobulin FW-GTC CTT GTG CTG GAC ACC GAC TAC A- 3′ and β-lactoglobulin REV-CAG GAC ACC GGC TCC CGG TAT ATG A- 3′ (Invitrogen-Thermo Fisher Scientific Inc., USA).

PCR-RFLP

The following steps were applied in the PCR reaction: denaturation at 95ºC for 2 minutes, 30 denaturation cycles at 95ºC for 1 minute, 30 cycles of hybridization at 57ºC (61ºC for β-lactoglobulin) for 30 seconds and 30 cycles of polymerization at 72ºC for 1 minute. The finish was followed by final elongation at 72 ° C for 7 minutes for κ-casein and 10 minutes for β-lactoglobulin. Identification of polymorphism in the κ-casein and β-lactoglobulin genes was done using a polymorphism based on the restriction fragment length polymorphism (RFLP) (Table 1). This method comprises the identification of polymorphism by treating PCR products with the appropriate restriction enzyme and by comparing the size of the strap on the agarose gel. The amplification products are purified by precipitation and treated with the restriction enzyme Hinf I (New England Biolabs Inc., USA) that specifically recognizes the 5'GANTC-3 'sequence, which includes polymorphism in the κ -casein gene and Hae III (New England Biolabs Inc., USA) that specifically recognizes the 5'GGCC-3 'sequence that encompasses polymorphism in the β-lactoglobulin gene according to the manufacturer's instructions. The polymorphism of restriction fragment length was analysed by agarose gel electrophoresis.

Table 1: Expected fragment sizes

Protein	Genotype	Fragment length
κ-casein	AA	156, 132 and 91
	AB	288, 156, 132 and 91
	BB	288 and 91
β-lactoglobulin	AA	144 and 108
	AB	144, 108, 74 and 70
	BB	108, 74 and 70

Statistical analysis

Statistical processing was performed in the SPSS Statistics for Windows, Version 23.0 program, which included determining the frequency of genotypes for both investigated genes. By using the $\chi 2$ test, the obtained frequencies were compared with frequencies based on the Hardy-Weinberg law on the equilibrium of the population, with the aim of confirming the hypothesis of the absence of equilibrium in the surveyed population, since the population was subject to prior selection.

RESULTS AND DISCUSSION

The study was carried out on 157 cows of Simmental breed located in the Toplica and Rasina district. Specific primers were used to detect genotypes for κ-casein and β-lactoglobulin. Polymorphism of restriction fragment size was analysed by agarose gel electrophoresis and three genotypes for both protein fractions were discovered. The length of the restriction fragments of κ-casein for the AA genotype was 156, 132 and 91 base pairs, for AB 288, 156, 132 and 91 base pairs, while for the genotype BB the restriction fragment was 288 and 91 base pairs. The results of the analysis of the κ-casein gene showed that the genotype AA was found in 53 cows, which makes a frequency of 33.80%, the AB genotype in 81 cows or 51.60% and the BB genotype in 23 cows or 14.60%. The A allelic frequency was 59.60%, while allele B had a frequency of 40.40%. (Table 2, Figure 1).

A slightly lower frequency of the AB genotype in their studies conducted on Simmental cows was determined by ĐEDOVIĆ *et al.* (2015), while the remaining two genotypes had a significantly different frequency compared to the results obtained. ILIE *et al.* (2010), in Simmental population in Romania, report in their study a frequency of 58.20% for the genotype AA, while the genotypes AB and BB show significantly lower frequency. The results most similar to this study are reported by FELENCZAK *et al.* (2007), for cows of the Simmental breed in Poland, while the established frequency of alleles A is lower than the frequency reported by IVANKOVIC *et al.* (2011), of 60.7-76.4% in selected cow breeds.

Table 2. Frequencies of κ -casein genotypes and the expected frequencies according to the Hardy-Weinberg equilibrium law

Genotype	Determined frequency, number	Determined frequency, %	Expected frequency, number	Expected frequency, %	Difference
AA	53	33.8	39.3	0.25	13.8
AB	81	51.6	78.5	0.50	2.5
BB	23	14.6	39.3	0.25	-16.3
Total	157	100.00			
$\chi2$=11.624	df=2		p value=0.003		

$\chi2$ = hi square test; df = degrees of freedom; p = significance

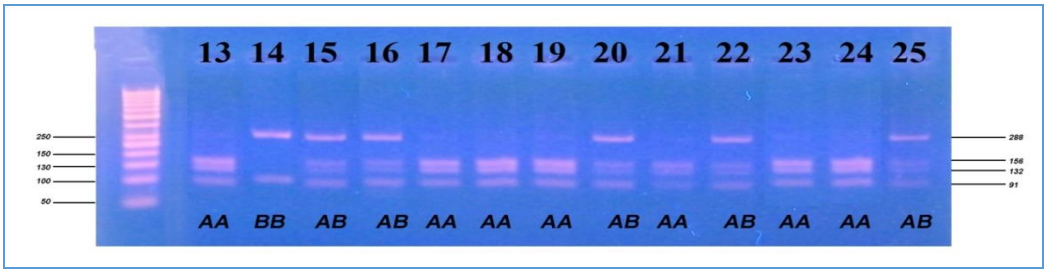

Figure 1. Determination of the k-casein gene variants using PCR-RFLP

Table 2 shows the frequencies of κ-casein genotypes and the expected frequencies according to the Hardy-Weinberg equilibrium law. Based on the results of $\chi2$ test and p values (p

<0.01), the frequencies determined differ statistically significantly from the frequency according to the Hardy-Weinberg equilibrium law, which confirmed the absence of equilibrium in the studied population since the population included in this study was subject to prior selection.

The length of the restriction fragments of β-lactoglobulin for the AA genotype was 144 and 108 base pairs, for AB 144, 108, 74 and 70 base pairs, while for the genotype BB the restriction fragment was 108, 74 and 70 base pairs. Table 3 lists the frequencies of genotypes and β-lactoglobulin alleles for the studied population of cows. The frequency obtained for the AA, AB and BB genotypes for β-lactoglobulin was 33.10%, 49.70% and 17.20%, respectively, which means that of total 157 cows, 52 had genotype AA, 78 genotype AB and 27 genotype BB. The frequency of alleles A and B resulting from the frequency of genotypes was 58.00% for allele A and 42.00% for allele B. As with κ-casein, same in β-lactoglobulin, it can be concluded that the frequency of the dominant alleles was higher in relation to the recessive alleles.

The determined values for the frequency of alleles B are lower than those stated by IVANKOVIC *et al.* (2011), while the frequencies of AA and AB genotypes are slightly lower than the frequencies indicated by FELENCZAK *et al.* (2007), and also these studies show no presence of the genotype BD, i.e. of allele D described in FELENCZAK *et al.* (2007). The obtained results are significantly different from the results achieved by CARO PETROVIĆ *et al.* (2017) conducted on a small population of cows of Simmental breed in Serbia.

Table 3 shows the frequency of genotypes and β-lactoglobulin alleles and the expected frequency according to the Hardy-Weinberg equilibrium law. Based on $\chi 2$ test results and p values ($p \leq 0.05$), the obtained frequencies statistically significantly differed from the frequency according to the Hardy-Weinberg equilibrium law, which confirmed the absence of equilibrium in the surveyed population.

Table 3. Frequencies of β-lactoglobulin genotypes and the expected frequencies according to the Hardy-Weinberg equilibrium law

Genotype	Determined frequency, number	Determined frequency, %	Expected frequency, number	Expected frequency, %	Difference
AA	52	33.1	39.3	0.25	12.8
AB	78	49.7	78.5	0.50	-0.5
BB	27	17.2	39.3	0.25	-12.3
Total	157	100.00			
$\chi 2$=7.968	df=2		p value=0.019		

$\chi 2$ = hi square test; df = degrees of freedom; p = significance

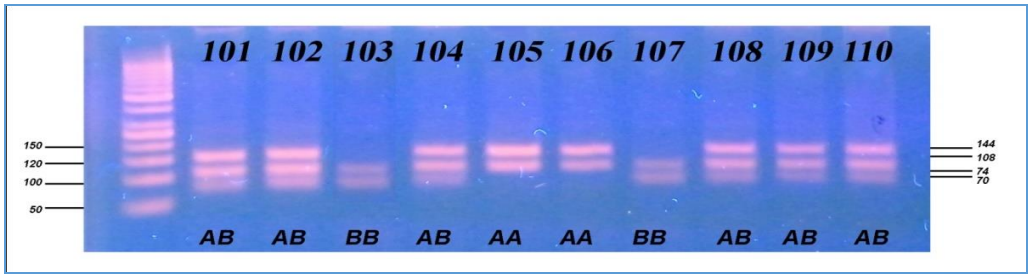

Figure 2. Determination of the β-lactoglobulin gene variants using PCR-RFLP

CONCLUSION

Determination of the frequencies of genotypes and alleles for κ-casein and β-lactoglobulin showed a higher frequency of allele A in both protein fractions. This is very important because by monitoring various forms of milk proteins and by determining genotypes and their incidence, we can increase the frequency of those genotypes that show positive effect on milk properties in certain populations of cows. By favouring suitable genotypes, or by selecting the parents with preferred genotypes, the faster genetic improvement of the population can be achieved, resulting in the improvements both in production and milk composition and reduction of losses in production.

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POLIMORFIZAN K-KAZEINA I B-LAKTOGLOBULINA KOD SIMENTALSE RASE GOVEDA U SRBIJI

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Izvod

Cilj ovog istraživanja bio je da se utvrdi frekvencija genotipova κ-kazeina i β-laktoglobulina u simentalskoj rasi goveda u Srbiji i da se uporedi sa frekvencijom po Hardy-Weinbergovom zakonu ravnoteže. Uzorci krvi uzeti su iz ukupno 157 krava simentalske rase u Topličkom i Rasinskom okrugu. Od 157 krava koje su uključene u ovo istraživanje, genotip AA *κ-kazeina* ustanovljen je kod 53 krave što čini frekvenciju od 33.80%, genotip AB imala je 81 krava ili 51.60% i genotip BB 23 krave ili 14.60%. Frekvencija alela A iznosila je 59.60%, dok je alel B imao frekvenciju od 40.40%. Što se tiče frekvencije genotipova i alela β-laktoglobulina za ukupnu ispitivanu populaciju krava dobijena za genotipove AA, AB i BB za β-laktoglobulin iznosila je 33.10%, 49.70% i 17.20%, što znači da su 52 imale genotip AA, 78 genotip AB i 27 genotip BB. Učestalost alela A i B koja proizilazi iz učestalosti genotipova, iznosila je 58.00% za alel A i 42.00% za alel B. Utvrđene frekvencije za obe frakcije proteina statistički su se značajno razlikovale od frekvencije po Hardy-Weinbergovom zakonu ravnoteže, čime je potvrđeno odsustvo ravnoteže u ispitivanoj populaciji.

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