Analysis of Beta-Lactoglobuline Gene (LGB) Polymorphism in Different Breeds of Bulls by High Resolution Melting

Martina Miluchová, Anna Trakovická, Michal Gábor

Slovak University of Agriculture in Nitra, 949 76–Nitra, Tr. A Hlinku, 2, Slovakia

Abstract

The goal of the paper was to identify β - lactoglobulin gene polymorphism in bulls. The β - lactoglobulin (LGB) is expressed in milk and is important in the evaluation of milk production potential and butterfat and protein content. LGB is localized on bovine chromosome 11. The AA genotype of LGB is associated with higher milk yield, the BB genotype with higher fat and casein content and is more desirable for cheese making. The material involved 46 bulls (Slovak spotted breed – 41 bulls, Pinzgau breed – 3 bulls and Holstein breed – 2 bulls). Bovine genomic DNA was isolated from sperm using commercial kit NucleoSpin Tissue and used in order to estimate LGB genotypes by means of PCR RFLP method and high resolution melting analysis (HRMA). In the population of Slovak spotted breed we detected all genotypes AA, AB and BB with frequency 0.3415, 0.4390 and 0.2195, subsequently. In Pinzgau breed was detected homozygote genotypes AA and BB with frequency 0.3333 and 0.6667. In Holstein breed was observed only heterozygote genotype AB with frequency 1.

Keywords: beta-lactoglobulin, bulls, HRMA

1. Introduction

Analysis of milk proteins polymorphism provides useful information to both the breeder and processor of milk. Many research reports have indicated that certain milk protein variants may be associated with milk production [1], milk composition [2,3] and cheese production [4,5].

β-Lactoglobulin was the first protein in which polymorphism was detected. By paper electrophoresis two distinct bands of β-lactoglobulin were observed, and named as β1 and β2 (A and B). Until now at least 12 variants are known for β-Lactoglobulin, out of which A and B variants are more frequent. β-lactoglobulin is the major whey protein in milk of cows and other ruminants e.g. deer, bison and buffalo, and in some non-ruminants such as pigs, horses, dogs, dolphins and whales. However, it is not an endogenous part in human milk [6].

β-lactoglobulin is an extremely stable protein which exists at the normal pH of bovine milk as a dimer with a molecular weight of 36,000 Daltons. It is a single chain polypeptide of 18 kDa comprising of 162 amino acid residues [7]. The bovine β-lactoglobulin A variant differs from B variant by two amino acids only i.e. aspartate-64 and valine-118. These amino acids are substituted by glycine and alanine respectively in the B variant. The biological functions of this protein are still not known. It could have a role in metabolism of phosphate and the transport of retinol and fatty acids [2].

The β - lactoglobulin (LGB) is expressed in milk and is important in the evaluation of milk production potential and butterfat and protein content. LGB is localized on bovine chromosome 11. The AA genotype of LGB is associated with higher milk yield, the BB genotype with higher fat and casein content and is more desirable for cheese making [8].

*Corresponding author: Martina Miluchová, Tel: 00421-6414290, Email:martina.miluchova@centrum.sk

2. Materials and methods

The material involved 46 bulls (Slovak spotted breed – 41 bulls, Holstein breed – 2 bulls, Pinzgau breed – 3 bulls). Bovine genomic DNA was isolated from sperm using NucleoSpin Tissue (Macherey-Nagel) and used in order to estimate LGB genotypes by means of PCR RFLP method and HRMA.

PCR RFLP

DNA primers described by Medrano and Aguilar-Cordova, (1990) [9] were used to PCR amplification: forward primer 5′ - TGT GCT GGA CAC CGA CTA CAA AAA G - 3′and reverse primer 5′- GCT CCC GGT ATA TGA CCA CCC TCT - 3′.

The PCR reaction elaborated by Medrano and Aguilar-Cordova, (1990) was modified.

The reaction mixture in the total volume 25 µl containing 50 ng DNA, 1 U Taq polymerase (FERMENTAS),1X PCR buffer (750 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)2SO_4$, 0.1% Tween 20), 1.5 mM $MgCl_2$, 200 µM dNTP, 5 pM of each primer. The following amplification parameters were applied: 94°C for 2 minutes followed by 35 cycles: 94°C for 60 seconds, 55°C for 60 seconds, 72°C for 60 seconds. The reaction was completed by the final synthesis: 72°C for 8 minutes.

The PCR products of 247 bp were digested with the *Hae*III restriction enzyme (Fermentas). Restriction digestion fragments were loaded on 3% agarose gel (Invitrogen) containing GelRed™ (Biotium) and the gel were analyzed in the UV rays.

HRMA

Optimization of the PCR conditions was carried out using the real-time thermocycler Rotor-Gene 6000® (Corbett Research, Australia). The reaction mixture in total volume 25 µl contained 1 × SensiMix™ HRM buffer (Bioline Reagents Ltd., UK) with 1 µl of EvaGreen® qPCR dye, 0.5 µM of each primer and 10 ng DNA template. The PCR conditions were 95°C for 10 min followed by 35 cycles of 95°C for 60 s, 55°C for 60 s (fluorescence acquisition on Green channel) and 72°C for 60 s. After real-time PCR, samples were heated to 95°C for 10 s, cooled to 45°C for 1 min, and melted from 82°C to 92°C, with the temperature increasing by 0.1°C increments with a 2 s hold at each step. HRM data were acquired by use of the HRM channel. HRM data were analyzed using Rotor-Gene 6000 Series Software version 1.7 (Corbett Research, Australia).

As standards for each genotype we used samples that were genotyping using by PCR RFLP method.

3. Results and discussion

PCR RFLP

*Hae*III digestion of the PCR products was analyzed by 3% agarose-gel electrophoresis. Allele A produced 148 and 99 bp fragments, and allele B produced 99 and 74 bp fragments as the PCR-RFLP.

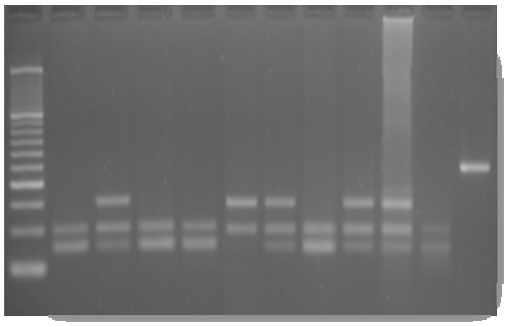

Figure 1. Representatively results of analysis PCR for LGB on 3% agarose gel
Line 1-marker 50 bp DNA Step Ladder (Promega); 2,4,5,8,11–genotype BB (99 bp, 74 bp); 3,7,9,10–genotype AB (148 bp, 99 bp, 74 bp); 6–genotype AA (148 bp, 99 bp); 12 –PCR product (247 bp)

HRMA

The HRM analysis was performed immediately after the pre-amplification in the real-time thermocycler. This analysis consisted of one cycle with increase in the temperature of 82°C to 92°C, where the change in fluorescence was measured at each 0.1°C rise for 2 s. The data obtained from amplification plots were used for a first correction of reaction quality by the comparative amplification function of the software Rotor-Gene 6000 Series Software version 1.7. Samples were considered to have failed if amplification had begun after 30 cycles.

For the screening of all genotypes were used the normalized high-resolution melt curves (Figure 2).

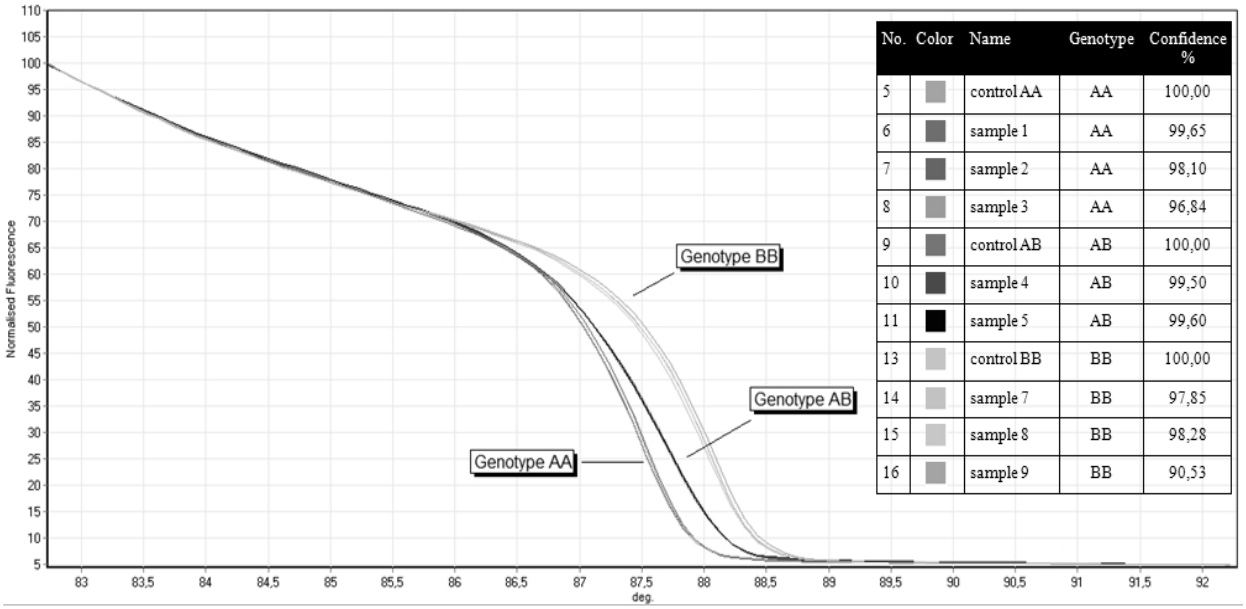

Figure 2. The high resolution normalized melting curves of a 247 bp PCR products for homozygous genotype AA, heterozygous genotype AB and homozygous genotype BB.

Normalization regions for the leading/trailing ranges were set at 83.99 - 84.99°C/90.81 - 91.81°C.

The difference graphs for HRMA normalized on genotypes AA, AB and BB is showed on Figure 3.

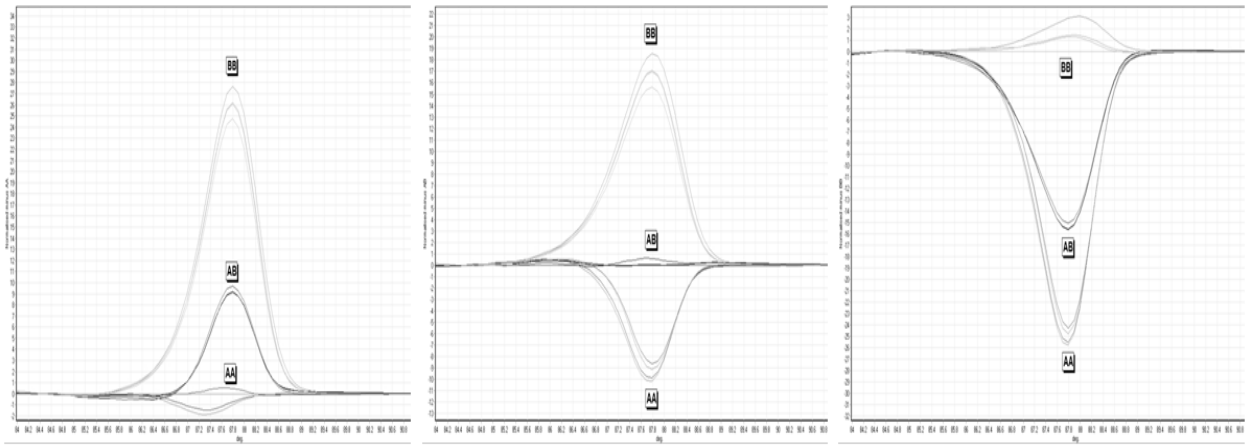

Figure 3. Difference graphs for HRMA normalised on genotypes AA, AB and BB

The negative dF/dT melt curves were generated from raw data of high resolution melting analysis. The value of peaks for the alelle A was 87.05 °C and for the alelle B was 80.1 °C. The negative dF/dT melt curves of heterozygous genotype were identified by peaks with value 87.5°C (Figure 4).

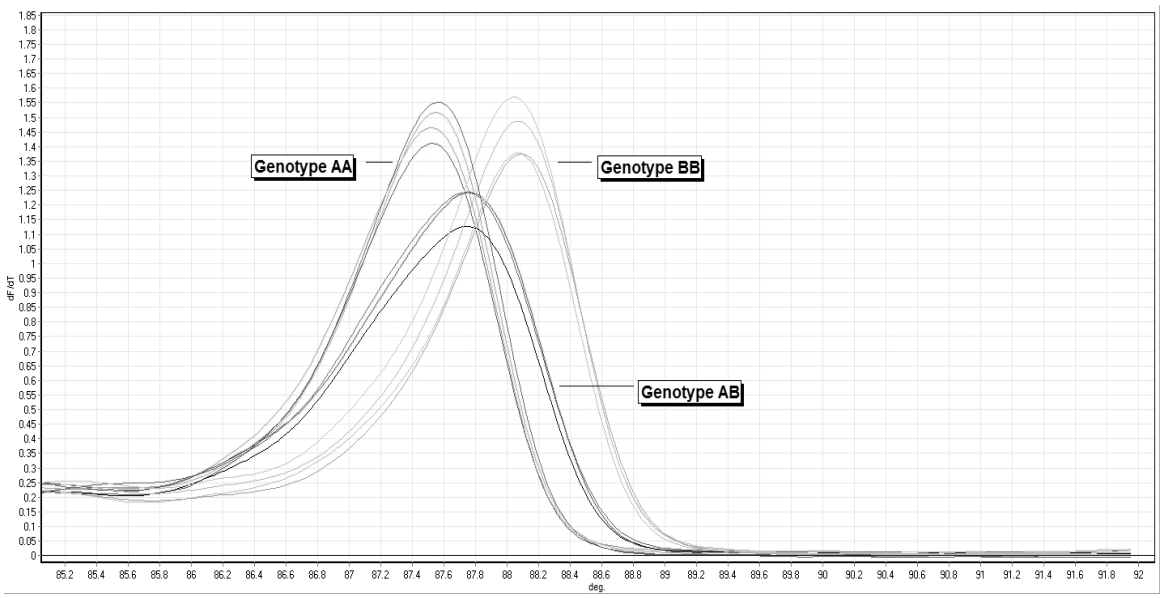

Figure 4. The negative derivative of fluorescence to temperature (- dF/dT) dissociation curve for homozygous genotype AA, heterozygous genotype AB and homozygous genotype BB.

In the population of Slovak Spotted breed we detected all genotypes AA, AB and BB with frequency 0.3415, 0.4390 and 0.2195, subsequently. In Pinzgau breed was detected homozygote genotypes AA and BB with frequency 0.3333 and 0.6667. In Holstein breed was observed only heterozygote genotype AB with frequency 1. In population of Slovak spotted breed was higher frequency of allele A (0.5610). In opposite, in population of Pinzgau breed was present higher frequency of the allele B (0.6667). The similar frequencies of the allele A and allele B were in population of Holstein breed. Genetic structure LGB gene of bulls is presented in table 1.

Frequencies of alleles in our population Slovak spotted breed were similar to those of LGB gene as reported by Kučerová et al. (2006) [10], who observed superiority of allele A (0.511) in Czech Fleckvieh breed. In opposite, Bulla et al. (2007) [11] present higher frequency of the allele B (0.73) in population of cattle. The higher frequency of the allele B (0.79) in population of Slovak Pinzgau cattle reported by Miluchová et al. (2010) [12].

Table 1. Genotype and allele frequencies of gene beta-lactoglobulin

BREED	BULLS	GENOTYPE FREQUENCIES			ALLELE FREQUENCIES	
		AA	AB	BB	A	B
Slovak Spotted breed	41	0.3415	0.4390	0.2195	0.5610	0.4390
Pinzgau breed	3	0.3333	0	0.6667	0.3333	0.6667
Holstein breed	2	0	1	0	0.5	0.5
TOTAL	46					

4. Conclusions

It may be concluded that in the population of Slovak Spotted breed is present LGB gene with a slight superiority of genotype AB and a superiority of allele A. In opposite, in population of Pinzgau breed was higher frequency of the genotype BB and allele B. In Holstein breed we detected only genotype AB that suggests the similar frequencies of the allele A and allele B. Genetic structure examined in population of Slovak spotted breed remained within the range quoted in literature for other cattle breeds.

Acknowledgements

This work has been supported by the grants: The Slovak Research and Development Agency under the contract No. LPP-0220-09 and the Excellence Center for Agrobiodiversity Conservation and Benefit project (ITMS: 26220120015) implemented under the Operational Programme Research and Development financed by the European Fund for Regional Development.

References

1. Bech, A.M., Kristiansen, K.R., Milk protein polymorphism in Danish dairy cattle and the influence of genetics variants on milk yield, J. Dairy Sci., 1990, 57, 53-63
2. Hill, J.P., Thresher, W.C., Boland, M.J., Creamer, L.K., Anema, S. G., Manderson, G., Otter, D. E., Paterson, G. R., Howe, R., Burr, R.G., Motion, R. L., Windelman, A., Wickham, B., The polymorphism of the milk protein β-lactoglobulin. In: Milk composition, production and biotechnology. Welch, et al. CAB International, Wallingford, UK., 1997, pp. 173-213.
3. Robitaille, G., Britten, M., Morisset, J., Petitclerc, D., Quantitative analysis of β-lactoglobulin A and B genetic variants in milk of cows β-lactoglobulin AB throught lactation, J. Dairy Res., 2002, 69, 651-654
4. Rachagani, S., Gupta, I.D., Gupta, N., Gupta, S.C., Genotyping of β-Lactoglobulin gene by PCR-RFLP in Sahiwal and Tharparkar cattle breeds, BMC Genetics, 2006, 31, pp. 1-4
5. Bujko, J., Optimalizácia genetického zlepšenia ukazovateľov mliekovej úžitkovosti v populácii slovenského strakatého plemena. Monograph, SPU, Nitra, 2011, 78 p., ISBN 978-80-552-0576-2
6. Hambling, S.G., McAlpine, A.S., Sawyer, L., β-lactoglobulin. In: Advanced Dairy Chemistry, vol.1 Proteins., Elsevier Applied Science, London, 1992, pp. 141–190
7. Creamer, L., Parry, D., Malcolm, G., Secondary structure of β-lactoglobulin B, Arch. Bioch. Bioph., 1983, 227, 98-105
8. Panicke, L., Freyer, G., Erhardt, G., Effects of milk protein genotypes on milk production traits. In: 48th Ann. Meet. of the Europ. Assoc. for Anim. Produkt. am 25.-28.08.1997 in Vienna. Wien, 1997
9. Medrano, J.F., Aguilar-Cordova, E., Polymerase chain reaction amplification of bovine β-lactoglobulin genomic sequences and identification of genetic variants by RFLP analysis, Anim. Bio-Technol., 1990, 1, 73-77
10. Kučerová, J., Matějíčková, A., Jandurová, O. M., Sørensen, P., Němcová, E., Štípková, M., Kott, T., Bouška, J., Frelich, J., Milk protein genes CSN1S1, CSN2, CSN3, LGB and their relation to genetic values of milk production parameters in Czech Fleckvieh, Czech J. Anim. Sci., 2006, 51, 241-247
11. Bulla, J., Chrenek, P., Michalcová, A., Krupová, Z., Szarek, J., Bulla, R., Ladyková, M., Adamczyk, K., Influence of κ-Casein and β -Lactoglobulin genes on milk yield, milk composition and technological properties of the different cattle breeds, Biotech, 2007, 54, pp. 60
12. Miluchová, M., Trakovická, A., Kadlečík, O., Kasarda, R., Hazuchová, E., HRM analýza beta-laktoglobulínového génu slovenského pinzgauského plemena, Acta fytotechnica et zootechnica, 2010, 13, pp. 79-82