Veterinarski Glasnik 2022, 76 (1), 17-24
UDC: 636.2:575
637.147.2
https://doi.org/10.2298/VETGL211125004R

Full Research Article

USE OF ALLELE SPECIFIC PCR TO INVESTIGATE THE PRESENCE OF β-CASEIN POLYMORPHISM IN HOLSTEIN-FRIESIAN COWS

Marko RISTANIC[1]*, Aleksandar NIKSIC[1], Mia NIKETIC[1], Stefan JELISIC[1], Milan Z RAJKOVIC[1], Uros GLAVINIC[1], Zoran STANIMIROVIC[1]

[1]University of Belgrade, Faculty of Veterinary Medicine, Department of Biology, Belgrade, Serbia

Received 25 November 2021; Accepted 21 February 2022
Published online: 01 April 2022

How to cite: Marko Ristanic, Aleksandar Niksic, Mia Niketic, Stefan Jelisic, Milan Z Rajkovic, Uros Glavinic, Zoran Stanimirovic. Use of allele specific PCR to investigate the presence of β-casein polymorphism in holstein-friesian cows. *Veterinarski Glasnik*, 2022. 76 (1): 17-24. https://doi.org/10.2298/VETGL211125004R

Abstract

Following the "one health" principle, we have conducted optimization of a protocol for β-casein genotyping in cattle in order to select cows with exclusively the A2A2 genotype. Gastrointestinal proteolysis of A1 β-casein in humans releases beta-casomorphin 7, which is believed to cause a number of diseases/conditions (diabetes mellitus type 1, ischemic heart disease, atherosclerosis, sudden infant death syndrome, autism, schizophrenia, gastrointestinal discomfort, and prolonged gastrointestinal passage time). On the contrary, A2 β-casein does not cause similar effects on human health, due to its different metabolism. DNA extraction was conducted from blood samples belonging to the laboratory archive of the Department of Biology, Faculty of Veterinary Medicine, University of Belgrade. Determination of genotypes was performed using the Allele Specific Polymerase Chain Reaction (AS-PCR) method. The amplification was preceded by determination of proper primer annealing temperature (65.50 °C), in order to ensure optimal genotyping results. The results obtained indicated a higher frequency of the A2 allele (0.56) compared to the A1 allele (0.44). Furthermore, in 7 out of 35 tested samples, the A1A1 genotype (20.00%) was found, in 17 samples, the A1A2 genotype (48.60%) was found, and in 11 samples, the A2A2 genotype (31.40%) was found. The molecular methods used ensured reliable β-casein genotyping that would enable selection of cows with the A2A2 β-casein genotype, implying production of

*Corresponding author – e-mail: mristanic@vet.bg.ac.rs

milk free of the undesirable A1 β-casein protein with all its potential negative impacts on human health.

Key words: β-casein, allele, AS-PCR, Holstein-Friesian, polymorphism

INTRODUCTION

Milk is one of the most common foodstuffs in the human diet, especially in children's diets. The biochemical composition of milk is nutritious thanks to its ratio of proteins, fats, and carbohydrates. Proteins are one of the most diverse groups of compounds in milk, and constitute 3.50% of cow's milk. Up to 80% of total milk proteins are caseins, and they consist of α-s1-(CSN1S1) casein, α-s2-(CSN1S2) casein, β-(CSN2) casein, and ϰ-(CSN3) casein, while the remaining 20% of milk proteins are whey proteins (α-lactalbumin and β-lactoglobulin) (Farrell et al., 2004). β-casein, which makes up around 25-30% of total proteins in cow's milk, is composed of a 209 amino acid long chain (Farrell et al., 2004).

β-casein is a highly polymorphic protein that has at least 12 different variants (Farell et al., 2004; Gallinat et al., 2013; Haq et al., 2014; Singh et al., 2015). The two most common forms of β-casein protein are types A1 and A2. It is believed that the A2 type is the original β-casein protein, and that the A1 type was a result of mutation during the domestication process of European cattle (Malarmathi et al., 2014). The main difference between A1 and A2 types of β-casein is in the amino acid composition at position 67 of the amino acid chain. In this position, histidine is present in the A1 type, while in the A2 type, proline occurs (Roginski et al., 2003). The occurrence of histidine in position 67 of the β-casein polypeptide chain causes the A1 type of β-casein to occur in milk. The importance of this amino acid difference is found in the gastrointestinal digestion of A1 type milk in the human body, as the opioid β-casomorphin 7 (BCM-7) is released (Kostyra et al., 2004). The effects of BCM-7 are manifested in various organ systems after it passes the chemoencephalic barrier and binds to μ/k/δ opioid receptors (Pal et al., 2015). Released BCM-7 increases the risk in consumers of disease development, such as type 1 diabetes mellitus, ischemic heart disease (Laugesen and Elliott, 2003), autism and schizophrenia (Cade et al., 2000), sudden infant death syndrome (Sun et al., 2003), atherosclerosis (Tailford et al., 2003) discomfort in the gastrointestinal system (Pal et al., 2015; Jianqin et al., 2016) as well as prolonged gastrointestinal passage time (Barnett et al., 2014). Considering all of the above, the aim of this study was to optimize a PCR method that could be used to select cows that will produce milk containing A2 β-casein protein exclusively and to utilize it for the "one health" concept.

MATERIALS AND METHODS

In this study, we used 35 randomly selected Holstein-Friesian (HF) cow blood samples originating from the laboratory archive of the Department of Biology, Faculty of

Veterinary Medicine, University of Belgrade. The blood was stored in sterile tubes with EDTA anticoagulant. DNA extraction was performed according to the manufacturer's instructions using the commercial DNA extraction kit MasterPure™ DNAPurification Kit for Blood Version II (Lucigen Corporation), adapted according to the instructions of the Animal Production and Health Laboratory, International Atomic Energy Agency (IAEA, Seibersdorf, Vienna). After DNA extraction, purity and DNA concentration measurements were conducted using a BioSpec-nano UV-VIS spectrophotometer. Extractions that had satisfactory DNA concentration and purity were used for further analysis. Amplification of the desired regions of the β-casein gene was performed using the Allele Specific Polymerase Chain Reaction (AS-PCR). The following primers, designed by Ganguly et al. (2013), were used for the amplification of a 244 base pair long DNA fragment of the β-casein gene: *Forward* primer with A nucleotide at the 3' end (IGBhF 5' CTT CCC TGG GCC CAT CCA 3') or *Forward* primer with C nucleotide at the 3' end (IGBpF 5' CTT CCC TGG GCC CAT CCC 3'), while the *Reverse* primer was mutual (IGBR 5' AGA CTG GAG CAG AGG CAG AG 3'). The role of the primer pairs IGBhF-IGBR and IGBpF-IGBR is to amplify specific regions of the β-casein gene, precisely, the alleles A1 or A2. The temperature regime for the PCR protocol consisted of the initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, primer annealing for 30 seconds and elongation at 72 °C for one minute. The final elongation was performed at 72 °C lasting one minute. Optimal primer annealing temperature was determined in gradient PCR (temperature range: 59 °C to 68 °C). The PCR mixture was prepared with a volume of 25 µl, which consisted of: 2.50 µl 10X KAPA Taq Buffer, 2 µl 25 mM $MgCl_2$, 0.50 µl 10 mM dNTP Mix, 1 µl of each primer (0.80 µM), 0.10 µl 5 U / µl KAPA Taq DNA Polymerase and 4 µl of DNA sample (100 ng/µl), according to manufacturer's instructions (Kappa Biosystems). Visualization of the PCR results was performed using the gel-electrophoresis method. For this purpose, 5 µl of PCR product was mixed with 6X DNA Loading Dye Buffer (Thermo Scientific), and the PCR products were then applied to a 2% agarose gel in which SimplySafeTM dye (EURx Ltd.) had been previously added. Electrophoresis was performed for a duration of 40 minutes and at a voltage of 100 V. The results were then observed under UV light and documented.

Genetic balance within the examined samples was estimated using the Hardy-Weinberg principle and analyzed with the χ^2 test.

RESULTS

In this study, the presence of A1 and A2 β-casein alleles was investigated from 35 randomly selected blood samples obtained from HF cows, using the AS-PCR method. In order to obtain an adequate PCR protocol, optimization of the primer annealing temperature was performed. The primer annealing temperatures were tested within the range from 59 °C to 68 °C. The results revealed that the temperature of 65.50 °C was optimal (Figure 1).

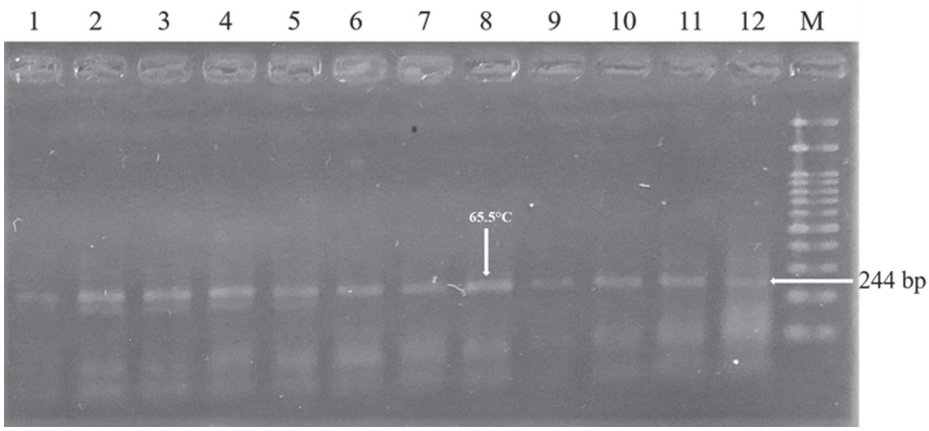

Figure 1. M - Ladder (100 bp); 1-12: PCR samples with different primer annealing temperatures

After the amplification of the target DNA regions, in which the optimized primer annealing temperature was applied, the resultant PCR products were visualized. Analyzing the PCR products on agarose gel, we observed the presence of amplicons specific for one or both alleles (depending on genotype of the animal) of the β-casein gene (Figure 2). The chosen method required analyzing each sample in duplicate, with one pair of primers (specific for each allele), individually. In this way, it was possible to distinguish three combinations of the β-casein alleles present (genotype variants). Samples that showed a specific band for the A1 allele (244bp) and absence of a band specific for A2 allele, were genotype A1A1, whereas samples that showed an electrophoretic band specific for the A2 allele (244 bp) and absence of a band specific for A1 allele were A2A2 genotype. Samples that showed bands specific for both alleles (244 bp in both replicates) were A1A2 genotype. Among the 35 analyzed samples, seven had the A1A1 genotype (20%), 17 samples originated from cows with the A1A2 genotype (48.60%), while genotype A2A2 was found in 11 samples (Table 1). Analysis of genotype and allele frequencies indicate that the values were in the Hardy-Weinberg equilibrium.

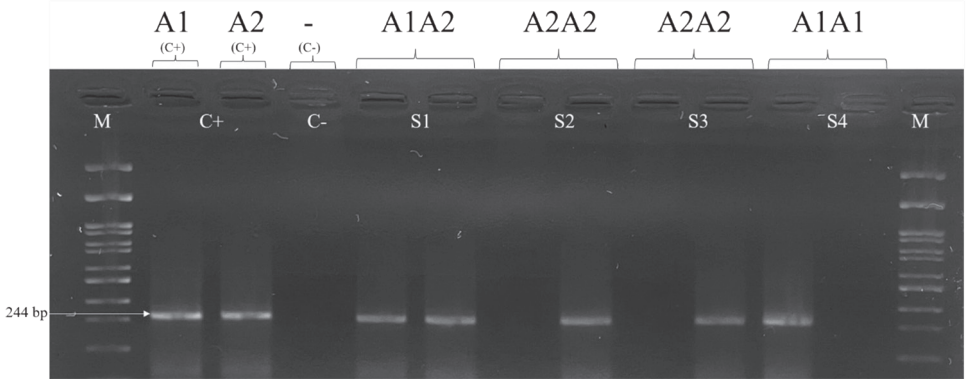

Figure 2. M - Ladder (100 bp); C+ Positive control; C- negative control; S1 - sample indicating the presence of A1A2 genotype; S2-S3 - samples indicating the presence of A2A2 genotype; S4 - sample indicating the presence of A1A1 genotype.

Table 1. Genotype and allele frequencies of the beta casein gene

Genotype of the β-casein	N	Genotype frequency (%)	Allele frequency	
			A1	A2
A1A1	7	20.00		
A1A2	17	48.60	0.44	0.56
A2A2	11	31.40		

DISCUSSION

Taking into account the effects of the A1 β-casein metabolite, BCM-7, a significant number of studies indicate a connection between A1 β-casein and its potential to cause various diseases or conditions in people that consume A1 milk (Ristanic et al. 2020). Given the fact that milk is one of the most common foodstuffs in the human diet, it is of great importance to study milk composition, especially its bioactive peptides such as BCM-7, which manifests negative effects on various organ systems. Therefore, adequate methods for identifying β-casein polymorphisms and assessing the presence of genotypic and allelic frequencies in cattle populations are needed (Ristanic et al., 2020). During our research, we determined the presence of genotype and allelic frequencies of the β-casein gene. Our results showed a higher frequency of the A2 allele (0.56) compared to the A1 allele frequency (0.44), which results from the higher frequencies of the A1A2 (54.72%) and A2A2 (33.02%) genotypes. Gustavsson et al. (2014) reported a significantly lower frequency of the A1 allele (0.26) compared to the A2 allele (0.61), as well as lower frequencies of genotypes A1A1 (6.70%) and A1A2 (33.20%) compared to A2A2 (37.70%) genotype. Similar results were reported by Kamiński et al. (2006), who determined a significantly lower frequency of allele A1 (0.40) compared to allele A2 (0.60), and a lower genotype frequency of A1A1 (11.19%) in comparison to the other two genotypes (A1A2 – 58.04% and A2A2 – 30.77%). The results obtained in our study are in accordance with the findings of various authors who performed similar research in HF cattle (Schopen et al., 2009; Visker et al., 2010; Molee et al., 2011; Massella et al., 2017; Ristanic et. al, 2020). The obtained results of allelic and genotype frequencies could be useful as a starting point for selecting cows with the desirable A2 allele, in order to encourage small and large dairy farmers to form herds that would produce A2 milk exclusively.

CONCLUSION

Having in mind the variety of PCR-based protocols for β-casein genotyping, which are mainly based on a Restriction Fragment Length Polymorphism PCR method, the AS-PCR used in our study offers a simple, quick, precise, and economical way of detecting β-casein polymorphism in cattle and does not require post-PCR enzyme

digestion. Utilization of this and similar methods in cattle breeding programs has created an opportunity for faster and easier selection of cattle with the A2A2 β-casein genotype, which can lead to the production of cow's milk without the undesirable A1 β-casein protein and all its potential negative effects on human health.

Acknowledgements

This study was supported and financed by the International Atomic Energy Agency (Contract No. 20774) which forms part of the IAEA Coordinated Research Project D31028.

Authors' contributions

RM and ZS designed the study and drafted the manuscript. RM, NA, NM, SJ and RZM performed laboratory analyses. UG and ZS interpreted the data, revised the manuscript and supervised the experiment.

Competing interests

The authors declare that they have no competing interests.

REFERENCES

Barnett M. P., McNabb W. C., Roy N. C., Woodford K. B., Clarke A. J. 2014. Dietary A1 β-casein affects gastrointestinal transit time, dipeptidyl peptidase-4 activity, and inflammatory status relative to A2 β-casein in Wistar rats. International Journal of Food Science, 65:720-727. https://doi.org/10.3109/09637486.2014.898 260.

Cade R., Privette M., Fregly M., Rowland N., Sun Z., Zele V. 2000. Autism and schizophrenia: Intestinal disorders. Nutritional Neuroscience, 3:57–72. https://doi.org/10.1080/1028415X.2000.11747303

Farrell H. M., Jimenez-Flores R., Bleck G. T., Brown E. M., Butler J. E., Creamer L. K., Hicks C. L., Hollar C. M., Ng-Kwai-Hang K. F., Swaisgood H. E. 2004. Nomenclature of the proteins of cows' milk—sixth revision. Journal of Dairy Science, 87:1641-1674. https://doi.org/10.3168/jds.S0022-0302(04)73319-6

Gallinat J. L., Qanbari S., Drögemüller C., Pimentel E. C. G., Thaller G., Tetens J. 2013. DNA-based identification of novel bovine casein gene variants. Journal of Dairy Science, 96(1): 699-709. https://doi.org/10.3168/jds.2012-5908

Ganguly I., Kumar S., Gaur G. K., Singh U., Kumar A., Kumar S., Mann S., Sharma A. 2013. Status of β-casein (CSN2) Polymorphism in Frieswal (HF X Sahiwal Crossbred) Cattle. International Journal of Biotechnology and Bioengineering Research, 4(3): 249-256

Gustavsson F., Buitenhuis A. J., Johansson M., Bertelsen H. P., Glantz M., Poulsen N. A., Månsson H. L., Stålhammar H., Larsen L. B., Bendixen C., Paulsson M. 2014. Effects of breed and casein genetic variants on protein profile in milk from Swedish Red, Danish Holstein, and Danish Jersey cows. Journal of Dairy Science, 97(6): 3866-3877. https://doi.org/10.3168/jds.2013-7312

Haq M. R., Kapila R., Shandilya U. K., Kapila S. 2014. Impact of milk derived β-casomorphins on physiological functions and trends in research: a review. International Journal of Food Properties, 17(8): 1726-1741. https://doi.org/10.1080/10942912.2012.712077

Jianqin S., Leiming X., Lu X., Yelland G. W., Ni J., Clarke A. J. 2015. Effects of milk containing only A2 beta casein versus milk containing both A1 and A2 beta casein proteins on gastrointestinal physiology, symptoms of discomfort, and cognitive behavior of people with self-reported intolerance to traditional cows' milk. Nutrition Journal, 15(1): 35. doi: 10.1186/s12937-016-0147-z

Kamiński S., Ruść A., Cieślińska A. 2006. A note on frequency of A1 and A2 variants of bovine beta-casein locus in Polish Holstein bulls. Journal of Animal and Feed Sciences, 15:195-98. doi: https://doi.org/10.22358/jafs/66892/2006

Kostyra E., Sienkiewicz-Szłapka E., Jarmołowska B., Krawczuk S., Kostyra H. 2004. Opioid peptides derived from milk proteins. Polish Journal of Food and Nutrition Sciences, 13:25-35.

Laugesen M, Elliott R. 2003. Ischaemic heart disease, Type 1 diabetes, and cow milk A1 beta-casein. The New Zealand Medical Journal, 116: 1168.

Malarmathi M., Senthil K. T., Parthiban M., Muthuramalingam T., Palanisammi A. 2014. Analysis of ß-casein gene for A1 and A2 genotype using allele specific PCR in Kangeyam and Holstein-Friesian crossbred cattle in Tamil Nadu. The Indian Journal of Animal Sciences, 43: 310-315. doi: 10.2478/acve-2020-0037

Massella, E., Piva S., Giacometti F., Liuzzo G., Zambrini A. V., Serraino A. 2017. Evaluation of bovine beta casein polymorphism in two dairy farms located in northern Italy. Italian journal of food safety, 6(3): 6904. doi: 10.4081/ijfs.2017.6904

Molee A., Boonek L., Rungsakinnin N. 2011. The effect of beta and kappa casein genes on milk yield and milk composition in different percentages of Holstein in crossbred dairy cattle. Animal Science Journal, 82: 512–516. https://doi.org/10.1111/j.1740-0929.2011.00879.x

Pal S., Woodford K., Kukuljan S., Ho S. 2015. Milk intolerance, beta-casein and lactose. Nutrients, 7(9): 7285-7297. https://doi.org/10.3390/nu7095339

Ristanic M., Glavinić U., Vejnović B., Maletić M., Kirovski D., Teodorović V., Stanimirović Z. 2020. Beta-casein gene polymorphism in Serbian Holstein-Friesian cows and its relationship with milk production traits. Acta Veterinaria, 70:497-510. doi: 10.2478/acve-2020-0037

Roginski H., Fuquay J. W., Fox P. F. 2003. Encyclopedia of dairy sciences, Volumes 1-4 pp. 2799

Schopen G. C. B., Heck J. M., Bovenhuis H., Visker M. H. P. W., van Valenberg H. J. F., van Arendonk J. A. M. 2009. Genetic parameters for major milk proteins in Dutch Holstein-Friesians. Journal of Dairy Science, 92: 1182-1191. https://doi.org/10.3168/jds.2008-1281

Singh L.V., Jayakumar S., Sharma A., Gupta S. K., Dixit S. P., Gupta N., Gupta S. C. 2015. Comparative screening of single nucleotide polymorphisms in β-casein and κ-casein gene in different livestock breeds of India. Meta gene, 4: 85-91. https://doi.org/10.1016/j.mgene.2015.03.005

Sun Z., Zhang Z., Wang X., Cade R., Elmir Z., Fregly M. 2003.: Relation of β-casomorphin to apnea in sudden infant death syndrome. Peptides, 24: 937-943. https://doi.org/10.1016/S0196-9781(03)00156-6

Tailford K. A., Berry C. L., Thomas A. C., Campbell J. H. 2003. A casein variant in cow's milk is atherogenic. Atherosclerosis, 170:13-19. https://doi.org/10.1016/ S0021-9150(03)00131-X

Visker M. H. P. W., Dibbits B. W., Kinders S. M., van Valenberg H. J. F., van Arendonk J. A. M., Bovenhuis H. 2011. Association of bovine β-casein protein variant I with milk production and milk protein composition. Animal Genetics, 42:212-218. https://doi.org/10.1111/j.1365-2052.2010.02106.x

UPOTREBA *ALLELE SPECIFIC PCR*-A U ISPITIVANJU PRISUSTVA POLIMORFIZMA β -KAZEINA KOD HOLŠTAJN-FRIZIJSKIH KRAVA

Marko RISTANIĆ, Aleksandar NIKSIĆ, Mia NIKETIĆ, Stefan JELISIĆ, Milan Z RAJKOVIĆ, Uros GLAVINIĆ, Zoran STANIMIROVIĆ

Kratak sadržaj

Kazeini čine 80% ukupnih proteina mleka, dok β-kazein i njegove varijante A1 i A2 zauzimaju značajno mesto u istraživanjima različitih autora. Metabolisanjem A1 tipa kod ljudi oslobađa se betakazomorfin-7, koji može da izazove niz oboljenja (dijabetes melitus tip 1, ishemijsku bolest srca, arterosklerozu, sindrom iznenadne smrti odojčadi, autizam, šizofreniju, nelagodnost u gastrointestinalnom sistemu i produženo vreme gastrointestinalne pasaže). Nasuprot tome, metaboliti A2 tipa β-kazeina, usled drugačijeg metabolisanja, ne izazivaju slične efekte po zdravlje ljudi. Cilj ovog istraživanja je bio optimizacija protokola za utvrđivanje polimorfizma β-kazeinskog gena u selekciji krava sa A2A2 genotipom, a prateći principe "jednog zdravlja". Za analize su korišćeni uzorci krvi iz laboratorijske arhive Katedre za biologiju FVM UB. Amplifikacija i detekcija aminokiselinske razlike između dva tipa β-kazeinskog proteina izvođena je metodom alelski specifične lančane reakcije polimeraze (*Allele Specific Polymerase Chain Reaction-AS-PCR*), kojoj je prethodila optimizacija temperature hibridizacije prajmera. Vizualizacija rezultata izvedena je pomoću gel-elektroforeze koja je omogućila uočavanje PCR amplikona specifičnih za oba različita alela β-kazeinskog gena, veličine 244 baznih parova. Analizama temperature hibridizacije prajmera, temperatura od 65,50 °C pokazala se kao optimalna. Dobijeni rezultati isptivanja su ukazali na veću frekvencu prisustva alela A2 (0,56) u odnosu na alel A1 (0,44) u ispitivanim uzorcima. Istovremeno od ukupno 35 ispitanih uzoraka kod 7 uzoraka krvi je bilo potvrđeno prisustvo genotipa A1A1 (20,0%), kod 17 uzoraka genotipa A1A2 (48,60%), odnosno kod 11 uzoraka genotipa A2A2 (31,40%). Korišćenjem ove i sličnih metoda u programima uzgoja goveda, mogla bi se izvršiti selekcija goveda koja imaju A2A2 genotip β-kazeina, što bi uslovilo proizvodnju kravljeg mleka bez neželjenog A1 β-kazeinskog proteina i svih njegovih implikacija po zdravlje ljudi.

Ključne reči: β-kazein, aleli, AS-PCR, holštajn-frizijska, polimorfizam