

## Evaluation of bovine beta casein polymorphism in two dairy farms located in northern Italy

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### Abstract

Bovine beta casein A1 is one of the most common variants in dairy cattle breeds; it is considered a risk factor in milk intolerance and in other important human diseases, because of the bioactive peptide beta casomorphin-7 (BCM7) produced by raw or processed A1-milk, but not by A2-milk, during digestion. The aim of this study was to perform a cheap and rapid method to investigate beta casein polymorphism in copious animals. The study included 2 dairy farms with a totally of 1230 cows. Beta casein genotypes were estimated evaluating Exon 7 region of bovine beta casein gene (CSN2) by sequences analysis. In the population included in the study 5 variants (A1, A2, B, F, I) and 13 genotypes (A1A1, A1A2, A1B, A1F, A1I, A2A2, A2B, A2F, A2I, BB, BF, BI, FI) were detected. The method showed high sensibility and specificity, resulted low-cost and few time consuming.

### Introduction

Cow's milk plays an important role in human diet. In milk, total proteins content is approximately 3.5% (Miller *et al.*, 2007) and the two major protein groups are represented by casein and whey proteins, occurring for about 80% and 20% respectively (Hoffman and Falvo, 2004). Bovine milk contains 4 caseins: alpha s1, alpha s2, beta and kappa, encoded by just as many genes (CSN1S1-CSN1S2-CSN2-CSN3, respectively), located on chromosome 6 (Rijnkels, 2002). Gamma-casein derives from degradation of beta casein (Kaminsky *et al.*, 2007). The beta casein constitutes up to 45% of bovine milk total casein and pre-

sents 12 genetic variants (A1, A2, A3, B, C, D, E, F, G, H1, H2 and I). In dairy cattle breeds variant A1 and A2 are the most common, B is less common and A3 and C are rare (Farrel *et al.*, 2004). Variant I derives from a mutation of variant A2 and has normally low frequencies. However in some breeds, i.e. Italian Holstein Fresian, Italian Red Pied and Dutsch Holstein Fresian this variant frequency can arise up to 0.120-0.190 (Jann *et al.*, 2002). Variant E was only found in the Italian Piemontese breed (Voglino, 1972), meanwhile the remaining variants have never been detected in European cattle breeds (Barroso *et al.*, 1999).

The beta casein variants (in particular A1, A2 and B) have received much attention from scientific community because of their influence on milk technological properties and on human health.

Beta casein variants play an important role in cheese yield/quality and their concentration is positively associated with good rennet properties of milk. More in detail, B variant is related to an increase in milk casein content, smaller casein micelle size, improving milk coagulation properties, and cheese yield in different cattle breeds (Dinc *et al.*, 2013; Ketto *et al.*, 2017). Also A1 variant improve rennet coagulation properties respect A2 variant, which has less influence on this technological property (Heck *et al.*, 2009). Meanwhile B variant is always characterised by small casein micelle size, promoting rennet coagulation, A1 and A2 variant can be associated with small or large casein micelle size (Day *et al.*, 2015).

Despite all the benefits related to milk consumption, such as anticarcinogenic, immunomodulatory, antimicrobial, anticarcinogenic, antihypertensive and hypocholesterolemic effects (Davoodi *et al.*, 2016), milk protein constitution can affect human health. The 2 main aspects concern: i) hypoallergenic properties of particular types of bovine milk containing potential allergens, mainly  $\alpha_{s1}$ -casein,  $\alpha_{s2}$ -casein,  $\beta$ -lactoglobulin protein, missing in human milk; ii) the release of peptides with biological function, resulting from enzymatic proteolysis of milk proteins during food processing or gastrointestinal digestion (Caroli *et al.*, 2009).

Regarding this last aspect, beta casomorphin-7 (BCM7) is an important bioactive peptide with strong opioid activity which can play a role in the development of some important human diseases. BCM-7 is released by proteolytic digestion of beta casein A1 and B, but not by A2. Specifically, it derives from a proteolytic split in position 67 of beta casein aminoacid chain, when

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histidine is present (variant A1 and B). Differently, A2 beta casein, which presents a proline in position 67 of the aminoacid chain, prevents BCM-7 production (Mishra *et al.*, 2009). A1 beta casein is also considered a risk factor for ischemic hearth disease, sudden infant death syndrome, aggravation of symptoms associated with schizophrenia and autism, type 1 diabetes and of milk intolerance (Kamiński *et al.*, 2006; Caroli *et al.*, 2009; Mishra *et al.*, 2009; Pal *et al.*, 2015). The role of A1 beta casein as undesirable variant has led to the attempt of selecting dairy cows, depending on their beta casein polymorphism. Programs of screening and crossbreeding were implemented in order to favour the desirable A2 variant among dairy breeds. In New Zealand this has led to the selection of cow herds producing milk with just A2 variant and, in 2003, to the launch on the market of A2 milk (Kamiński *et al.*, 2007).

The hypothesis that consumption of A1 beta casein increases the risk of several human diseases is very intriguing and interesting. However, the undesirable role of A1 beta casein is not yet confirmed and requires further investigations. The importance for human health to study and to determine beta casein polymorphism in dairy cattle has led to the development of many investigation methods, based on microarray or DNA amplification (i.e. restriction fragment length polymorphism-PCR; bidirectional allele specific-PCR; multiplex-PCR) and sequencing, focusing on nucleotide chain mutations. The aim of this study was to describe the occurrence and the frequencies of beta casein variants

and genotypes in a large number of cows living in north Italy, proposing an easy and cheap method, with high sensibility and specificity, to identify polymorphic variants of beta casein located on Exon 7, in order to highlight the animals with A2A2 genotype, as a valiant alternative to genotyping methods adopted until now.

## Materials and Methods

From October 2016 to May 2017, 1230 blood samples were collected from dairy cattle (1226 Holstein Fresian and 4 Braunvieh) coming from two different breeding farms located in Emilia-Romagna region (northern-Italy). DNA was extracted by using DNeasy Blood and Tissue kit (Qiagen, Germany). The region Exon 7 of CSN2 was amplified by PCR according to Dai *et al.* (2016). Briefly, PCR amplification was performed in a reaction volume of 25  $\mu$ L containing 12.5  $\mu$ L REExtract-N-Amp PCR ReadyMix (Sigma-Aldrich, USA), 10 mmol L<sup>-1</sup> primers (Exon VII forward: 5'-AGGCAACTCAGGAAGAGGTG-3'; Exon VII reverse: 5'-ATCTC-CACGGGTAAGCCTAGA-3') and 2.5  $\mu$ L DNA. The following amplification parameters were applied: an initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds, elongation at 72°C for 1 minute and a final extension at 72°C for 7 minutes. The amplified products were analysed by electrophoresis on 1% agarose gel containing Sybr Safe DNA gel staining (Thermo Fisher Scientific, USA) and run in 0.5X TBE buffer at 100 V for 30 minutes. PCR product of 995 bp was then sequenced by an external service (Eurofins Genomics, Ebersberg, Germany).

The obtained sequences were aligned with the reference sequence of bovine beta casein (GenBank, sequence number: X14711) using the software BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and then analysed using the same software with the addition of the Mega6 one (<http://www.megasoftware.net>).

During sequences analysis 7 mutations of the reference sequence were considered: the first, in position 8101, is characterized by an A (adenine) for A1, B, F alleles and by a C (cytosine) for A2, A3, H2, I alleles; the second, in position 8115, is characterized by a C (cytosine) for A1, A2, A3, B, F, H1, I and by a G (guanine) for H2 allele; the third, in position 8163, is characterized by a C (cytosine) for A1, A2, A3, B, F, H2, I alleles and by an A (adenine) for H1 allele; the fourth, in position 8178, is characterized by an A (adenine) for A1, A2, A3, B, F, H1 alle-

les and by a C (cytosine) for H2, I alleles; the fifth, in position 8219, is characterized by a C (cytosine) for A1, A2, B, F, H1, H2, I alleles and by an A (adenine) for A3 allele; the sixth, in position 8267, is characterized by a C (cytosine) for A1, A2, A3, F, H1, H2, I alleles and by a G (guanine) for B allele; the seventh, in position 8356, is characterized by a C (cytosine) for A1, A2, A3, B, H1, H2, I alleles and by a T (thymine) for F allele (Chessa *et al.*, 2007; Caroli *et al.*, 2009).

In some cases the analysis of the single ferogram and the evaluation of peaks related to each mutation were necessary to define the presence of the same alleles or of two different alleles.

The method proposed in this paper was validated using five samples coming from five dairy cows, previously genotyped as A1A1, A1A2, A2A2, A1B, A2B. These samples, together with bovine beta casein reference sequence, were always included in sequences analysis in order to establish the reliability of the results.

In addition, sequences analysis results were occasionally validated by Fragment Length Polymorphism PCR of CSN2, which identified A1A1/A1B-A1A2/A2B-A2A2 genotypes, following the protocol described by Mishra *et al.* (2009).

## Results

In the population included in the study the most common allelic variant of beta casein was A2, followed by A1, with frequencies of 0.546 and 0.371, respectively. Allelic variants B and I were less common with percentages of 0.050 and 0.027, meanwhile allelic variant F was rare (0.006).

Variants A3, H1 and H2 were not found.

We detected 13 genotypes: 3 homozygote genotypes (A1A1, A2A2, BB) and 10 heterozygote genotypes (A1A2, A1B, A1F, A1I, A2B, A2F, A2I, BF, BI, FI). The heterozygote A1A2 genotype was the most frequent with a percentage greater than 0.400. The homozygote genotypes A2A2 and A1A1 resulted in percentages of 0.301 and 0.139, respectively. All the other genotypes showed percentages lower than 0.052, with BF and FI genotypes as the less frequent. Detailed genotypes and alleles frequencies in the examined population are presented in Table 1.

## Discussion

The method proposed for the evaluation of beta casein polymorphism results a simple typing system, which allows genotyping of the most beta casein variants. The relatively low cost and fast implementation, combined with the high sensibility and specificity, make it suitable for routinely and large-scale screening of beta casein variants in dairy cattle breeds. However, this method cannot identify C, D, E and G variants. Despite this limit, it is important to consider that E variant was identified only in Piemontese breed and, as C and G variants, is rare; while D variant was only found in *Bos indicus* (Caroli *et al.*, 2009).

This paper provides further knowledge about occurrence and frequencies of beta casein variants and genotypes in dairy cattle in Italy. Desirable A2 allele showed higher frequency (0.546) than A1 allele (0.371). These results correspond to high frequencies of heterozygous genotype A1A2 and homozygous genotype A2A2 (0.403 and

**Table 1. Alleles and genotypes frequencies observed in the examined population.**

Allele	Allele frequency	Genotype	Genotype frequency
A1	0.371	A1A1	0.139
A2	0.546	A1A2	0.403
A3	0	A1B	0.036
B	0.050	A1F	0.005
F	0.006	A1I	0.020
H1	0	A2A2	0.301
H2	0	A2B	0.052
I	0.027	A2F	0.006
		A2I	0.028
		BB	0.004
		BF	0.001
		BI	0.004
		FI	0.001

0.301 respectively). Our results are in accordance with those reported by Kaminsky *et al.* (2007) that show frequency of A1 and A2 alleles between 0.402-0.472 and 0.470-0.598 respectively in Holstein Fresian cows. Only in the USA this trend is overturn with frequencies of A1 allele higher than A2 allele (0.310-0.660 and 0.240-0.620 respectively) (Kaminsky *et al.*, 2007).

Allele B frequency was 0.050, according to Kaminsky *et al.* (2007), which reported frequency of B variant between 0.010 and 0.107 in Holstein Fresian cows. These results correspond to low frequency of genotypes A1B-A2B (<0.052) and BB-BF-BI (<0.004)

Variant F is rare and there are not available data about its frequency in dairy breeds. In our study F variant was found at low frequency (0.006).

Variant I shows a frequency <0.030. A previous study reported that in different European cattle breeds variant I frequency was usually <0.030, but it showed higher frequency in some breeds like Italian Red Pied (0.140) and Italian Holstein Fresian (0.120) (Jann *et al.*, 2002). Also in Dutsch Holstein Fresian variant I frequency reached 0.192 (Visker *et al.*, 2010)

Alleles A3, H1 and H2 were not found. The absence of A3 variant observed in this study is not surprising considering the low frequency in all breeds analysed (<0.005) whenever presents (Jann *et al.*, 2002; Chessa *et al.*, 2013; Dai *et al.*, 2016). Regarding to H1 and H2 variants there are not available data about their frequencies.

## Conclusions

In recent years the genetic polymorphism of beta casein has received considerable interest in dairy industry and animal breeding because of their involvement in various aspects of human nutrition and health. In this paper the application of a quite easy and cheap method to identify the most frequent genotypes of bovine beta casein is described. These features make it useful for screening of large dairy cattle animals and therefore suitable to be used in routine beta casein genotype evaluation.

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