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Modification of a previously patented method to unequivocally score A2-like and A1-like bovine β-casein variants

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ABSTRACT

A growing interest in the production and commercialization of A2 cow's milk has been observed in many countries in the last few years due to the beneficial properties for human health attributed to A2 β-casein variant. Methods of varying complexity and different equipment requirements have been proposed for the determination of the β-casein genotype of individual cows. We proposed herein a modification of a previously patented method based on an amplification-created restriction site PCR followed by restriction fragment length polymorphism analysis. This method allows to identify and differentiate A2-like from A1-like β-casein variants, after differential endonuclease cleavage flanking the nucleotide that determines the amino acid at position 67 of β-casein. The advantages of this method are that it:

- enables to unequivocally score A2-like as well as A1-like β-casein variants,
- can be performed at low cost in simply equipped molecular biology laboratories, and
- can be scaled up to analyze hundreds of samples per day.

For these reasons, and based on the results obtained from the analysis carried out in this work, it showed to be a reliable method for the screening of herds to selective breeding of homozygous cows and bulls for A2 or A2-like alleles.

Specifications table

Subject area:	Biochemistry, Genetics and Molecular Biology
More specific subject area:	Amplification-created restriction site PCR followed by restriction analysis technique.
Name of your method:	Method to unequivocally discriminate A2-like and A1-like bovine β-casein variants.
Name and reference of original method:	C.N. McLachlan, Breeding and milking cows for milk free of β-casein A1, United States Patent 7,094,949 (2006).
Resource availability:	N.A.

Method details

Beta casein, along with alpha s1, alpha s2, and kappa caseins are phosphoproteins synthesized in the mammary gland in response to lactogenic hormone and other stimuli [1]. The caseins in bovine milk constitute a family of polymorphic proteins in which different

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genetic variants can be distinguished within each group. For β-casein, 12 variants (A1, A2, A3, B, C, D, E, F, G, H1, H2, and I) were described in different breeds of cattle, with A1 and A2 being the most frequent forms [2]. The difference between these last two variants is due to a single nucleotide polymorphism (SNP rs43703011) consisting of the replacement of a cytosine (in variant A2) by an adenine (in variant A1) in exon 7 of the beta casein (*CSN2*) gene, which results in replacement of proline by histidine amino acid at position 67 of the protein, respectively. This amino acid substitution produces conformational changes in the secondary protein structure and therefore affects its proteolysis during enzymatic digestion in the intestine and/or milk technological processing [3]. Proteolytic cleavage of the A1 variant, with a histidine at position 67, leads to the release of a seven-amino acids peptide named β-casomorphine 7 (BCM-7), with a strong opioid activity [4]. Regarding BCM-7 production, some casein variants ("A1-like variants", B, C, F and G) behave similarly to A1, while others ("A2-like variants", A3, D, E, H1, H2, I) behave like A2 [5]. Several studies reported a correlation between the presence of BCM-7 in milk or the "A1-milk" consumption and an increased risk for type 1 diabetes [6], ischemic heart disease [7,8], sudden infant death syndrome [9], and increased gastrointestinal inflammation [10]. Other studies proposed an impact of "A1-milk" consumption on autism, schizophrenia, and even milk allergy. Despite these reported correlations, the negative role of the β-casein variant A1 remains somewhat controversial [11]. On the other hand, the consumption of β-casein A2 was reported to have a lower risk compared to β-casein A1 against digestive problems, type 1 diabetes, cardiovascular diseases, and neurological diseases (for reviews see [12,13]). Anyway, there has been increasing interest in the production and processing of A2-milk in recent years, driven by consumers' concerns and commercial reasons. In this context, many farmers around the world, motivated by the selective propagation of desirable allelic variants, switched to A2-milk production by breeding only homozygous β-casein A2 or A2-like cows.

Genetic restructuring of herds to produce only A2-milk implies genotyping the *CSN2* gene in bulls and cows to discriminate between carriers of A1- and A2-like variants. Different analytical techniques were applied to achieve this goal: allele-specific PCR [14], amplification-created restriction site (ACRS)-PCR followed by restriction analysis [15], ligation detection reaction-universal array assay [16], high-resolution melting (HRM) and rhAmp® SNP genotyping [17], among others. Herein we present a method to discriminate between A1- and A2-like β-casein variants by DNA analysis consisting in a modification of the ACRS-PCR method patented by McLachlan [15]. This method involves an artificial creation of a restriction site by directed mutagenesis in the PCR step, followed by restriction fragment length polymorphism (RFLP) analysis. In the original method, the amplified region has a restriction site (artificially created) only in A2 or A2-like alleles, generating hence two fragments after the endonuclease digestion, while it remains uncut in A1 or A1-like alleles. So, incomplete digestion of the PCR products (due to inadequate reaction conditions in terms of buffer or temperature or insufficient digestion time) or absence of cleavage given by technician error (omission of enzyme addition), could lead to an incorrect allele assignment. To overcome this method limitation and unequivocally score A2-like as well as A1-like alleles, we propose a modification of McLachlan's method with the inclusion of an internal (positive) control for digestion in the amplified PCR product. As there is no restriction site for the same enzyme that differentiates A1 and A2-like variants in the amplicon or even flanking it, we use a restriction site for another enzyme, which is compatible in buffer and digestion conditions with the former. This internal control for digestion is present in A1- as well as in A2-like variants. This easy-to-perform and low-cost routine method can be carried out in any molecular biology laboratory and requires no special equipment except for a thermal cycler, an electrophoresis system and an UV trans-illuminator.

Samples

DNA was extracted from hair root samples taken from the tail of Holstein cows using the MasterPure DNA Purification Kit (Epicentre, Madison, WI, USA) and between 30 and 40 hair follicles of each animal, following the supplier's protocol. Besides, genomic DNA from bulls was obtained from semen straws using a standard phenol-chloroform-based extraction method. The quantity and quality of the extracted DNA was evaluated by measuring the absorbance at 260 nm and computing the A260/A280 and A260/A230 ratios, respectively, using a NanoDrop^{TM} 1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). Obtained A260/A280 ratios varied between 1.69 and 2.33 while A260/A230 ratios were in the range of 1.08 - 2.41. DNAs were diluted in distilled water, their concentration adjusted to 40 ng/μl and kept at 4 ˚C until the PCR reaction.

Genotyping

The genotyping was done by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). The PCR was designed to amplify a product of 316 bp containing the locus in exon 7 of *CSN2* gene determining the amino acid at position 67 of the β-casein. The amplicon considered includes the region amplified by McLachlan's method. The chosen primers were forward 5′-TGTGAAGAAAGTGGGTTAATGAGAA-3′ and reverse 5′- GAGTAAGAGGAGGGATGTTTTGTGGGAGGCTCT-3′. The latter creates a DdeI restriction site and was previously described by McLachlan (US Patent 7,094,949 B2, SEQ ID NO: 1) [15], while the former was designed based on the bovine *CSN2* gene sequence (GenBank Accession No. X14711.1) using Primer3 software, available online [18]. Default settings of Primer3 software were used, with the exception of those for primer size, which was imposed to be between 18 and 25 bp. The product size range was set at 250 to 500 bases. Both primers were supplied from Genbiotech (Buenos Aires, Argentina). The quality and specificity of the primer sequences were checked using the online tools OligoAnalyzer IDT (https://www.idtdna.com/calc/analyzer) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). PCR reactions were set up for a final volume of 15 μl containing a final concentration of 0.1 U/μl of Paq5000® DNA Polymerase (Agilent Technologies, Stratagene, Santa Clara, CA, USA), 1X Paq5000^{TM} Reaction Buffer (Agilent Technologies, Stratagene, Santa Clara, CA, USA), 200 μM of each dNTP (GE Healthcare, Buckinghamshire, UK), 0.5 μM of each forward and reverse primers, and approximately 40 ng of DNA template.

Fig. 1. Schematic representation of fragments produced from 316 bp amplicons after endonuclease digestion a) in A1 or A1-like alleles, and b) in A2 or A2-like alleles.
Primers used for PCR reactions are highlighted in gray. Restriction sites for HinfI and DdeI endonucleases are showed in bold blue and red fonts, respectively. The SNP that determines the amino acid at position 67 of the β-casein protein and distinguishes A1- from A2-like alleles based on the band pattern obtained after restriction enzyme digestion is highlighted in yellow. The nucleotide that creates a restriction site by directed mutagenesis in the PCR step is underlined (it is a cytosine in GenBank Accession No. X14711.1 reference sequence).

Amplification was performed in 96-well Thermoquick PCR plates (Greiner Bio-One, Stonehouse, USA) in an Eppendorf Mastercycler thermal cycler (Eppendorf AG, Hamburg, Germany). Conditions for amplification were: initial denaturation at 94 ˚C for 4 min, followed by 35 cycles of 30 s at 94 ˚C (denaturation), 30 s at an annealing temperature of 58 ˚C, and 45 s at 72 ˚C (extension). Reactions were finished with a 72 ˚C 8-min extension. Controls for PCR reactions were: non-template control (NTC, in which the DNA was replaced with sterile bidistilled water) and three positive controls, one for each of the three possible genotypes (DNA samples coming from homozygous A2A2, heterozygous A1A2, and homozygous A1A1 bulls, selected based on information from the bull sale catalog). Moreover, each PCR reaction included two random replicate samples. After the amplification, both duplicated samples and the NTC were used to check for PCR efficiency by analyzing the amplicons in 2.5% agarose gels stained with ethidium bromide along with a 50 bp DNA ladder (PB-L Productos Bio-Lógicos, Argentina). Electrophoresis was performed in 1X TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0) at a constant voltage of 100 V for 40 min using an ENDURO^{TM} horizontal gel box (Labnet International, Inc.) and visualized under UV illumination. If the NTC showed no amplification and duplicated samples presented the expected 316 bp amplicons, the rest of the PCR reaction was subjected to restriction enzyme digestion. Amplicons were digested overnight at 37 ˚C using a mix of DdeI (NEB, New England BioLabs, UK) and HinfI (NEB, New England BioLabs, UK) endonucleases at a final concentration of 0.23 U/μl each, in 1X rCutSmart buffer. Under these reaction conditions, digestion was complete. After incubation, the digested products were loaded on 4% agarose gels with ethidium bromide containing 1X TBE buffer and ran in 1X TBE buffer at 80 V for 5 min, followed by 100 V for 1 h, along with a 50 bp DNA ladder (PB-L Productos Bio-Lógicos, Argentina). The gel was visualized and examined for different band patterns under UV light and images were captured with an Axygen Gel Documentation System-BL (Axygen, Corning, NY, USA).

HinfI enzyme cleaves the 316 bp amplicon in two fragments of 178 and 138 bp in every sample, serving as an internal positive digestion control. The 178 bp fragment constitutes a positive control for the addition of the digestion reaction mix on the PCR products. In A2-like alleles, the DdeI enzyme produces a second cut on the 138 bp fragment, yielding 103 bp and 35 bp fragments. The expected small restriction fragment of 35 bp was not properly visible on the gel but was irrelevant for β-casein variants discrimination anyway. In A1-like alleles, the 138 bp fragment lacks the restriction site for DdeI, remaining uncut. Therefore, the proposed method enables the three genotypes (A1A1, A1A2, and A2A2) to be unequivocally discriminated, based on the band pattern visualized on the gel. DNA homozygous for the A2 (or A2-like) allele result in two fragments of 178 and 103 bp, DNA homozygous for the A1 (or A1-like) allele yield two fragments of 178 and 138 bp, and DNA heterozygous A1A2 (or A1-like/A2-like), result in three fragments of 178, 138 and 103 bp (Fig. 1). Some unspecific, larger size, fragments are also visualized on the gel, but those neither affect β-casein variants discrimination. Among 1152 Holstein cows screened, 602 were homozygous for the A2 (or A2-like) allele, 54 were of homozygous for the A1 (or A1-like) allele, and 496 were heterozygous. An image of an agarose gel showing the pattern of bands obtained after the electrophoretic run of DNA amplified but not digested, DNA from animals with genotypes A1A1, A1A2, and A2A2 for β-casein, and the non-template control of PCR reaction, can be appreciated in Fig. 2.

Method validation

The reliability of the proposed method was confirmed by the analysis of DNA from two bulls with known β-casein genotypes and direct sequencing of the 316 bp amplicons obtained after amplification. The animals were selected based on information from the bull sale catalog so that they had the genotypes A1A1 and A1A2 for β-casein.

The amplification products from those bulls were purified using the ADN Puriprep-GP kit (Inbio Highway, Tandil, Argentina), according to the manufacturer's recommendations. Bidirectional Sanger sequencing was carried out using the same PCR primers as used for the PCR reaction previously described and the BigDye® Terminator chemistry (Applied Biosystems, Foster City, USA). DNA sequences were obtained by capillary electrophoresis on an ABI 3130xl sequencer (Applied Biosystems, Foster City, USA), and later assembled, analyzed and visually checked using the Staden package software [19].

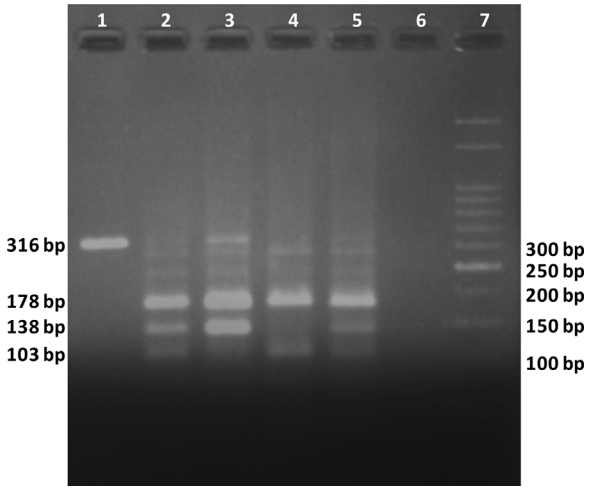

Fig. 2. Band pattern after electrophoresis.
The agarose gel image shows the pattern of bands obtained after the electrophoretic run of DNA amplified but not digested (316 amplicon, lane 1), DNA from a cow with unknown genotype (which resulted to be heterozygous for A1-like and A2-like alleles, lane 2), DNA from animals with genotypes A1A1 (lane 3), A2A2 (lane 4), and A1A2 (lane 5) for β-casein, and the non-template control of PCR reaction (lane 6) along with the DNA 50 bp ladder (lane 7).

As expected, the sequence obtained for the A1A1 genotype was homologous to the portion of the bovine *CSN2* gene sequence (GenBank Accession No. X14711.1) corresponding to the amplified region, showing an adenine for the SNP rs43703011. While the sequence obtained for the A1A2 genotype was homologous to the same reference sequence deposited in GenBank, and presented an M (A/C) at the locus for the SNP rs43703011 which determines the amino acid in position 67 of the protein and hence differentiates between A1 and A2 β-casein variants. These sequencing results demonstrate the specificity of the PCR reaction.

In conclusion, the proposed method proved to be reliable for identifying and differentiating A2-like from A1-like β-casein variants, enabling the determination of the β-casein genotype of individual animals. Furthermore, it can be scaled up to analyze hundreds of samples per day. We suggest that this is a worth considering method for simply equipped laboratories that aim to genotype β-casein variants to provide advice to producers on the selection and breeding of A2 cows and bulls for production of pure A2 milk.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

María Agustina Raschia: Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Project administration. **María Eugenia Caffaro:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Project administration, Validation. **Úrsula Amaranta Rossi:** Validation. **Mario Andrés Poli:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Data availability

The authors do not have permission to share data.

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