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New high-sensitive rhAmp method for A1 allele detection in A2 milk samples

Rodrigo Giglioti[a,*], Gunta Gutmanis[a], Luciana Morita Katiki[a], Cintia Hiromi Okino[b], Márcia Cristina de Sena Oliveira[b], Anibal Eugênio Vercesi Filho[a]

[a] Instituto de Zootecnia (IZ), Rua Heitor Penteado, 56, Zip Code 13380-011 Nova Odessa, SP, Brazil
[b] Embrapa Pecuária Sudeste (CPPSE), Rod. Washington Luiz, Km 234, CP 339, Zip Code 13560-970 São Carlos, SP, Brazil

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ABSTRACT

Cows' milk may contain two types of β-casein: A1 and A2. A1 digestion is associated with the release of β-casomorphine-7 peptide, which can cause adverse gastrointestinal effects. Two methods – high-resolution melting (HRM) and rhAmp® SNP genotyping – were developed to identify the β-casein gene (*CSN2*) A1 and A2 alleles directly in milk. DNA milk samples from 45 animals were examined and 10 samples were also sequenced to confirm the accuracy of the assays. The analytical sensitivities of both strategies for A1 allele identification were evaluated by testing decreasing dilutions of A1 allele DNA copies (500 – 5 copies) in the A2 sample. The limits of detection for A1 in A2 samples were 10% (100 copies) and 2% (10 copies) for HRM and rhAmp, respectively. Both techniques were specific, differentiating between A1 and A2 alleles. However, we recommend rhAmp genotyping testing over HRM because of its enhanced sensitivity for A1.

1. Introduction

Milk and dairy products, especially those derived from cows' milk, are important sources of protein worldwide, providing important macro- and micronutrients, with their consumption continuing to increase worldwide (Jianqin et al., 2016; Kamiński, Cieślińska, & Kostyra, 2007; Kamiński, Ruść, & Cieślińska, 2006; Visioli & Strata, 2014). Milk from dairy cows consists of two large groups of proteins: caseins and whey proteins. The caseins constitute 80% of bovine milk proteins and have four forms: α-S1- (*CSN1-S1*, 39–46%), α-S2- (*CSN1-S2*, 8–11%), β- (*CSN2*, 25–35%) and κ-casein (*CSN3*, 9–15%), that occur in the approximate proportions 4:1:4:1, respectively (Grosclaude, 1988; Visser, Slangen, & Rollema, 1991).

There are 13 genetic variants of β-casein, the most frequent of which found in cattle are A1 and A2 variants (Farrell et al., 2004). Differences between A1 and A2 β-casein are due to a mutation at amino acid 67 (proline at A2 to histidine at A1) (Bonfatti, Di Martino, Cecchinato, Vicario, & Carnier, 2010). Histidine present at position 67 in A1 β-casein results in the cleavage of the preceding seven amino acid residues, generating the peptide β-casomorphin-7 (βCM-7) (Jinsmaa & Yoshikawa, 1999). Compared to A2, the CSN2 A1 variant is more easily hydrolysed by digestive enzymes present in the human gastrointestinal tract, due to weaker binding between the isoleucine and histidine, releasing the peptide β-casomorphine-7 (βCM-7) (De Noni & Cattaneo, 2010; De Noni, 2008; Nguyen, Johnson, Busetti, & Solah, 2015). βCM-7 obtained after A1 milk digestion may be associated with increased risk of diseases, such as Type 1 diabetes mellitus (Chia et al., 2017), autism (Jarmołowska et al., 2019; Whiteley et al., 2010), schizophrenia (Severance et al., 2010) and increased gastrointestinal inflammation (Jianqin et al., 2016). Milk containing A2 β-casein is associated with reduced gastrointestinal symptoms of milk intolerance and enhanced lactase activity compared to conventional milk containing A1 β-casein (He, Sun, Jiang, & Yang, 2017).

No existing reports have found the A2 allele of β-casein to be related to adverse health problems. Even under physiological conditions, the opioid peptide βCM-7 is reportedly released from A1 milk digestion, but not from A2 (Brooke-Taylor, Dwyer, Woodford, & Kost, 2017). According to Brooke-Taylor et al.'s study, this difference is associated with slower gastrointestinal transit and consequently increased gastrointestinal transit times. The authors also demonstrated that according to some human groups, the A1-derivative peptide βCM-7 is pro-inflammatory. Thus, the consumption of A2 milk may be considered as a dietary alternative for individuals exhibiting gastrointestinal discomfort owing to the consumption of cow's milk, unrelated to lactose

* Corresponding author.
E-mail addresses: giglioti@iz.sp.gov.br (R. Giglioti), gunta@iz.sp.gov.br (G. Gutmanis), lmkatiki@iz.sp.gov.br (L.M. Katiki), cintia.okino@embrapa.br (C.H. Okino), marcia.sena-oliveira@embrapa.br (M.C. de Sena Oliveira), anibal@iz.sp.gov.br (A.E. Vercesi Filho).

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intolerance.

Given the relationship between *CSN2* A1 and A2 as well as their impacts on human health, these two variants have been extensively studied in dairy herds (Gustavsson et al., 2014; Kamiński, Ruść, & Cieślińska, 2006; Keating, Smith, Ross, & Cairns, 2008; Lien et al., 1999; Rangel et al., 2017; Royo, del Cerro, Vicente, Carballal, & Roza-Delgado, 2014; Visker et al., 2010). However, there remains no standard method for genotyping these alleles of the *CSN2* gene directly in milk. Moreover, most research is strictly related to genotyping the animals, whereas the present study is focused on the identification of A1 presence/contamination in A2 milk samples.

High-resolution melting (HRM) is a post-PCR method that allows detection of subtle sequence polymorphisms, by monitoring the melting behaviour of PCR amplicons using a highly saturating fluorescent dye (Wittwer, Reed, Gundry, Vandersteen, & Pryor, 2003). Alternatively, the rhAmp® method uses RNase H2 to activate primers after they have become successfully bound to their target sites, reducing primer dimer formation and improving the specificity of the reaction (Dobosy et al., 2011). For the rhAmp assay, the biallelic discrimination is achieved through the competitive binding of two allele-specific forward primers, one labelled with fluorescein amidite (FAM) dye and the other with Yakima Yellow (YY) dye. Therefore, this study seeks to develop and compare these different rapid methods for the genotyping of A1 and A2 alleles directly in milk samples.

2. Material and methods

2.1. Experimental samples and processing

Blood and milk samples were provided by a commercial farm (Descalvado, Brazil; 21°54′14″S, 47°36′10″W). Twenty Holstein cows that had previously been genotyped by DNA sequencing as A1 or A2 (n = 10) were used during the development of the assays. Milk samples were also individually collected from another 35 Holstein cows. In addition, five samples of A2A2 commercial milk were obtained from five different milking batches and were processed.

All procedures were approved by the Animal Science Institute Ethical Committee for Animal Experimentation (CEUA-IZ) in accordance with the ethical principles and guidelines of animal experimentation adopted by the Brazilian College of Experimentation (Process Number 238-16).

2.2. DNA extraction

DNA extraction was performed using an Easy-DNA™ kit (Cat. no. K1800-01—Protocol #1—Small Blood Samples and Hair Follicles; Invitrogen, Carlsbad, US), as recommended by the manufacturer. Briefly, 60 µL of whole blood was mixed with 100 µL of Solution A and incubated at 65 °C for 10 min. 40 µL of Solution B and 140 µL of chloroform were added, mixed and centrifuged at 20,817 × g and 4 °C (all centrifugation steps were conducted at the same velocity and temperature) for 15 min. The supernatant was separated in new microtubes and 445 µL of Tris-EDTA and 5 µL of mussel glycogen were added and mixed. One millilitre of absolute ethanol was added, mixed and incubated in ice for 30 min. Microtubes were centrifuged for 15 min. The supernatant was discarded and 500 µL of ethanol 80% was added, mixed and centrifuged for five minutes. The supernatant was then discarded and centrifuged for three minutes. Residual ethanol was removed by pipetting and pelleted DNA was eluted in 60 µL of Tris-EDTA. Milk samples were also pre-processed according to Reale, Campanella, Merigioli, and Pila (2008) method, with some modifications. 20 mL phosphate-buffered saline (PBS) was added to 20 mL fresh milk and centrifuged at 1500 × g at 4 °C for 20 min. Additional washing was performed by adding 10 mL PBS and the samples were centrifuged at 1500 × g at 4 °C for 10 min. The obtained pellet was resuspended in 60 µL PBS and then subjected to DNA extraction using the protocol adopted for blood samples. The quantity and the purity of the extracted DNA were estimated by spectrophotometric readings at 260 nm and ratios of 260/280 nm, respectively. The DNA concentrations of all tested samples were adjusted to 5 ng/µL.

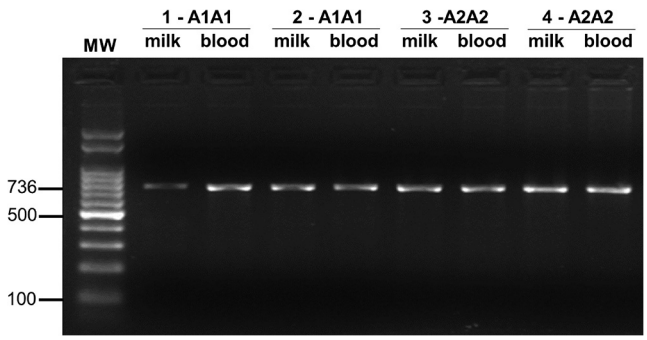

Fig. 1. Representative results obtained by electrophoresis of eight PCR products of *CSN2* gene including A1 and A2 mutations (genotypes previously identified by sequencing), from milk and blood samples (four animals). The expected PCR fragment size was 736 bp. MW = Molecular-weight size marker 100 bp.

2.3. DNA sequencing

A set of primers (Supplementary Table 1) were constructed from sequences flanking a fragment of 736 nucleotides located in the bovine β-casein encoding gene (*CSN2* gene; ID: 281099). The primers were designed using PrimerQuest software (http://www.idtdna.com/Primerquest/Home/Index). The specificity and the quality of the sequences were tested using the online tools NetPrimer (http://www.premierbiosoft.com/netprimer/), OligoAnalyzer IDT (https://www.idtdna.com/calc/analyzer) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGETYPE=BlastSearch&LINKLOC=blasthome).

The DNA extracted from milk and blood samples (n = 20) was submitted to PCR reactions. The assays were performed for a final volume of 50 µL using 5 µL of Platinum™ Taq DNA Polymerase High Fidelity (5 U/µL; Invitrogen), 1.5 µL of $MgSO_4$ (Invitrogen), 0.2 µL of Taq DNA Polymerase PCR Buffer 10 × [(600 mM Tris–SO_4 (pH 8.9), 180 mM $(NH_4)_2SO_4$; Invitrogen)], 1 µL of 10 mM dNTP mix (Sigma–Aldrich, St. Louis, USA), 2 µL of each 10 µM forward and reverse primer (Supplementary Table 1) and 2 µL of DNA (10 ng). The PCR thermocycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s (denaturation), 55 °C for 45 s (annealing) and 72 °C for 1 min (extension), with a final extension at 72 °C for 10 min. The amplification products were submitted to electrophoresis in 2.0% agarose gel, stained with ethidium bromide and visualised under ultraviolet light. The PCR products were purified using a PureLink® PCR purification kit (Invitrogen) according to the manufacturer's recommendations. PCR products from the same animal (blood and milk) were pooled and the DNA was sequenced using the same PCR primers. The sequencing reaction was performed using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, USA) and then analysed by an ABI Prism 3730XL DNA analyser (Applied Biosystems). The DNA sequences obtained were aligned using CLUSTAL/W software (Thompson, Higgins, & Gibson, 1994) and compared to those already deposited in GenBank. The contig sequences were also evaluated by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and deposited in GenBank (access numbers: MK426695.1 and MK426696.1).

Following DNA sequence confirmation, two synthetic DNA gBlocks® Gene Fragments (Integrated DNA Technologies [IDT], Coralville, USA) were produced, each containing the specific sequence of either allele (A1 and A2) used as controls for the HRM and rhAmp assays.

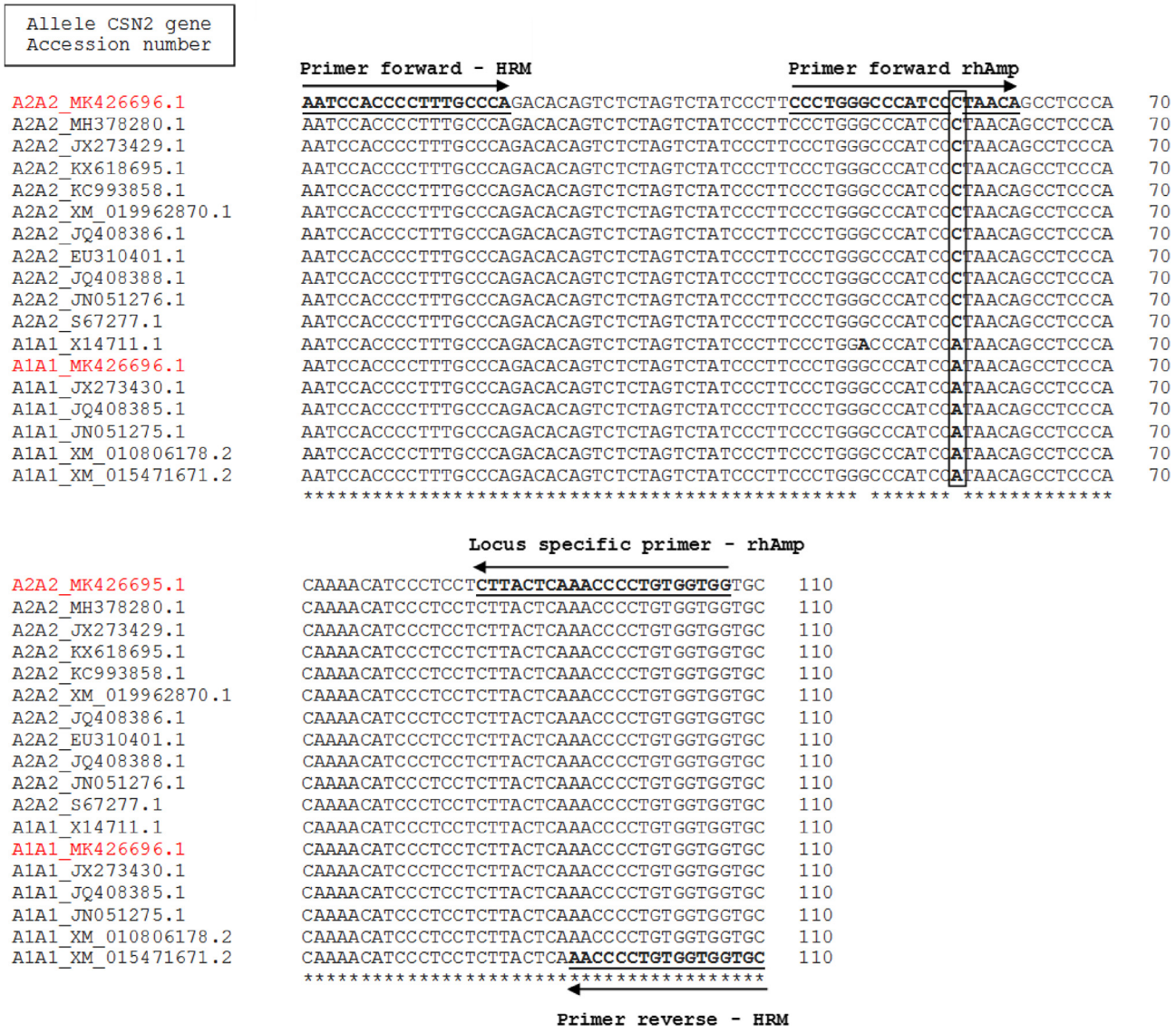

Fig. 2. Nucleotide alignment of eighteen sequences (β-casein *CNS2*) and primers from HRM (110 bp) and rhAmp (65 bp) analysis. The arrows and bold indicate the location of the qPCR primers/probes and the rectangle indicates the nucleotide difference between the analyzed sequences: the presence of adenine determines the A1 allele and the presence of cytosine determines A2. Another single mutation was found in 49 nucleotide position of X14711.1 sequence. The sequences deposited in the study are A2A2_MK426695.1 and A1A1_MK426696.1.

2.4. Real-time PCR assay followed by HRM analysis

A set of primers flanking the SNP (single nucleotide polymorphism) associated with A1/A2 genotypes were constructed as described in Section 2.3, using the sequences MK426695.1 and MK426696.1.

A real-time PCR (qPCR), followed by HRM analysis, was performed in 10 µL reaction volumes using a Rotor-Gene Q thermocycler (Qiagen, Venlo, Netherlands). Each reaction contained 6.4 µL of sterile water, 2 µL of 5 × HOT FIREPol EvaGreen® HRM mix (Solis Biodyne, Tartu, Estonia), 0.3 µM of each primer (βqPCRF and βqPCRR) and 1 µL of DNA (5 ng). A negative template control was included in each PCR run. The qPCR was performed using the following conditions: initial denaturation at 95 °C for 12 min, followed by 35 cycles of denaturation (95 °C for 15 s), annealing (65 °C for 20 s) and extension (72 °C for 20 s). After amplification, HRM analysis for genotyping was performed during dissociation curves from 70 to 92 °C in 0.2 °C increments, rising at 0.1 °C/2 s. Standard samples (containing 500 DNA copies per reaction) of each genotype were included in all qPCR runs: A1A1 (A1 synthetic DNA or A1A1 animal sample previously sequenced), A2A2 (A2 synthetic DNA or A2A2 animal sample previously sequenced) and A1A2 (50% A1 synthetic DNA + 50% A2 synthetic DNA or 50% A1A1 animal sample previously sequenced + 50% A2A2 animal sample previously sequenced).

The reactions were carried out in 100 µL microtubes (PCR® strip tubes and caps; Product no. PCR-0104-C; Axygen®, New York, USA) using a Rotor-Gene Q (Qiagen) equipped with a 72-well rotor. Rotor-Gene Q software was used to analyse and determine the genotypes, using a confidence value of ≥ 90%.

2.5. rhAmp® SNP genotyping

The rhAmp® Genotyping Design Tool (IDT; https://eu.idtdna.com/site/order/designtool/index/GENOTYPING_PREDESIGN) was used to design the primers, resulting in the assay ID: CD.GT.QWDC2715.6 (Supplementary Table 1). The rhAmp genotyping assay was performed by following the manufacturer's instructions. Briefly, 2 µL (10 ng) of DNA sample was mixed with 5.3 µL of rhAmp Genotyping Mix [1 mL of rhAmp Genotyping Master Mix 2× (Cat. no. 1076017; IDT) and 50 µL

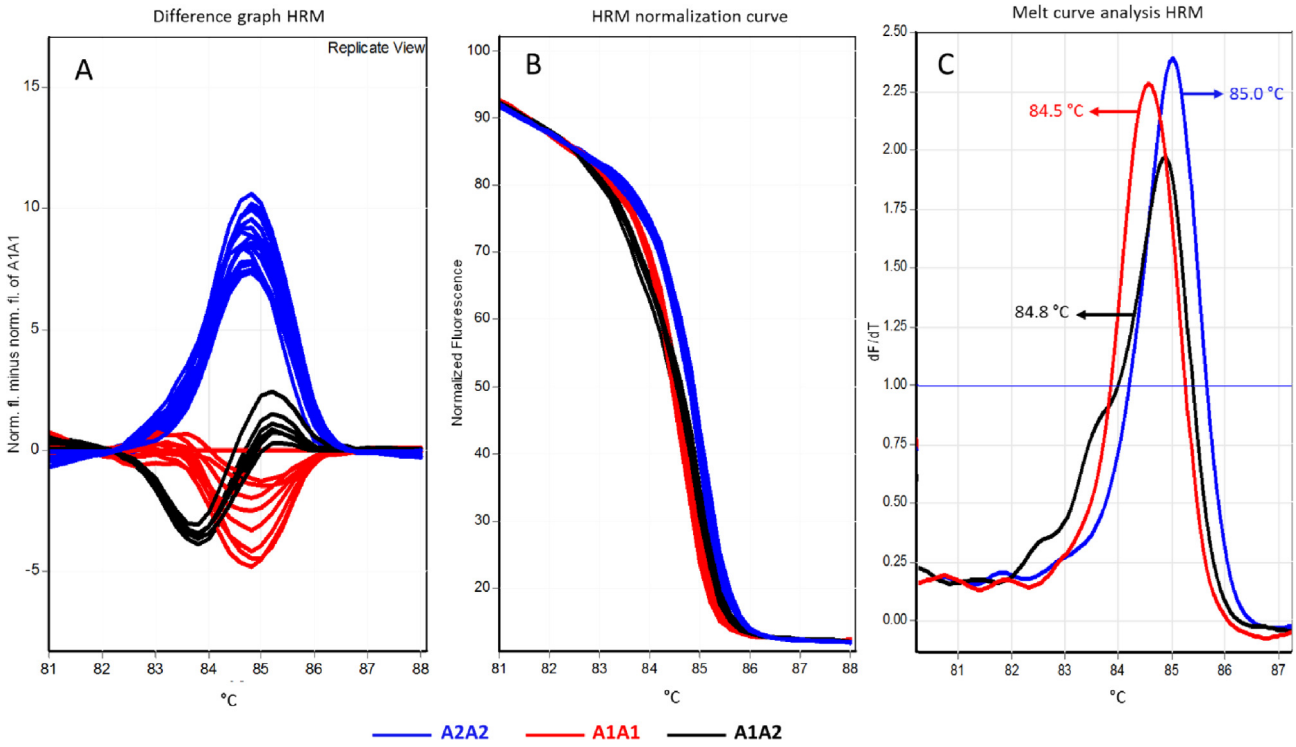

Fig. 3. Results of High-resolution melting curve analysis in bovine milk samples. The mutant type A1A1 was used as a baseline in the Normalized and shifted melting curves. A – HRM Analysis data difference plot: the results were presented as normalized fluorescence minus normalized fluorescence of A1A1 standard sample, all clusters are easily discerned; B – Raw data melt curves: the plot demonstrates the sharp decrease in fluorescence: homozygous A1A1 (red line), heterozygous A1A2 (black line) and homozygous A2A2 (blue line). C – Derivative melt curves: the differences between the three genotypes are also discriminated by changes in temperature values of melting peak (˚C) and a different pattern is observed in the heterozygote. There was no difference between the results obtained from synthetic DNA and standard samples (previously sequenced). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of rhAmp Reporter Mix (Cat. no. 1076028; IDT)] and 0.5 μL of custom rhAmp SNP assays (IDT), attaining a final volume of 10 μL. The same standard samples and negative template controls used in the HRM analysis were also included in this assay. The thermal cycling conditions were 95 ˚C for 10 min (enzyme activation), followed by 40 cycles of denaturation (95 ˚C for 10 s), annealing (60 ˚C for 30 s) and extension (68 ˚C for 20 s), plus a final heat inactivation (99.9 ˚C for 15 min). Rotor-Gene Q operating software was used to determine allelic discrimination and to analyse and ascertain the genotypes, using a confidence value of ≥90%.

2.6. A1 allele identification in A2 samples

The limits of detection of both developed qPCR methods were determined by testing tenfold serial dilutions of synthetic DNA from A1A1 or A2A2 genotypes (from 4×10^{9} DNA copies to 4×10^{-1} DNA copies). Briefly, decreasing concentrations of A1 in A2 synthetic DNA were tested as follows: 50% (500 copies), 40% (400 copies), 30% (300 copies), 20% (200 copies), 10% (100 copies), 5% (50 copies), 2% (10 copies) and 1% (5 copies). These tests were also performed using DNA samples from previously genotyped animals, replacing A1 and A2 synthetic DNAs by A1A1 and A2A2 DNA samples, respectively.

3. Results

3.1. DNA sequencing

The PCR products from the primer set βPCRF and βPCRR were specific, as observed in Fig. 1. All A1A1- and A2A2-sequenced DNA samples showed 100% of homology compared to respective A1A1 and A2A2 sequences previously deposited in GenBank, with the exception of the A1A1_X17411.1 sequence, which presented another mutation at nucleotide position 49 (Fig. 2).

3.2. HRM genotyping

All tested samples were successfully genotyped for the three possible genotypes (A1A1, A1A2 and A2A2) via HRM analysis. The three genotypes were easily identified, especially due to their different melting temperature peaks (Fig. 3c) and their positive or negative peaks of normalised fluorescence minus fluorescence of A1A1 control (Fig. 3a). For each genotype, the same patterns were observed between different milk and blood samples from different animals and controls (gBlocks and sequenced samples). The genotype frequencies of evaluated animals for A1A1, A1A2 and A2A2 were 36%, 31% and 33%, respectively. The five commercial samples from five different milking batches presented the A2A2 genotype.

3.3. rhAmp genotyping

The rhAmp assays successfully genotyped all of the milk samples evaluated. Fig. 4a and b present the amplification curves from two channels: green (FAM) and yellow (YY). The green channel (FAM) detected the A2 allele (wild allele), while the yellow channel (VIC) detected the A1 allele (mutant allele). Both signals detected the heterozygous variant (A1A2). As observed in the HRM analyses, the standard samples and the milk samples presented the same patterns as each other (Fig. 4).

3.4. A1 allele identification in A2 samples

Regarding the sensitivities of the methods developed for the

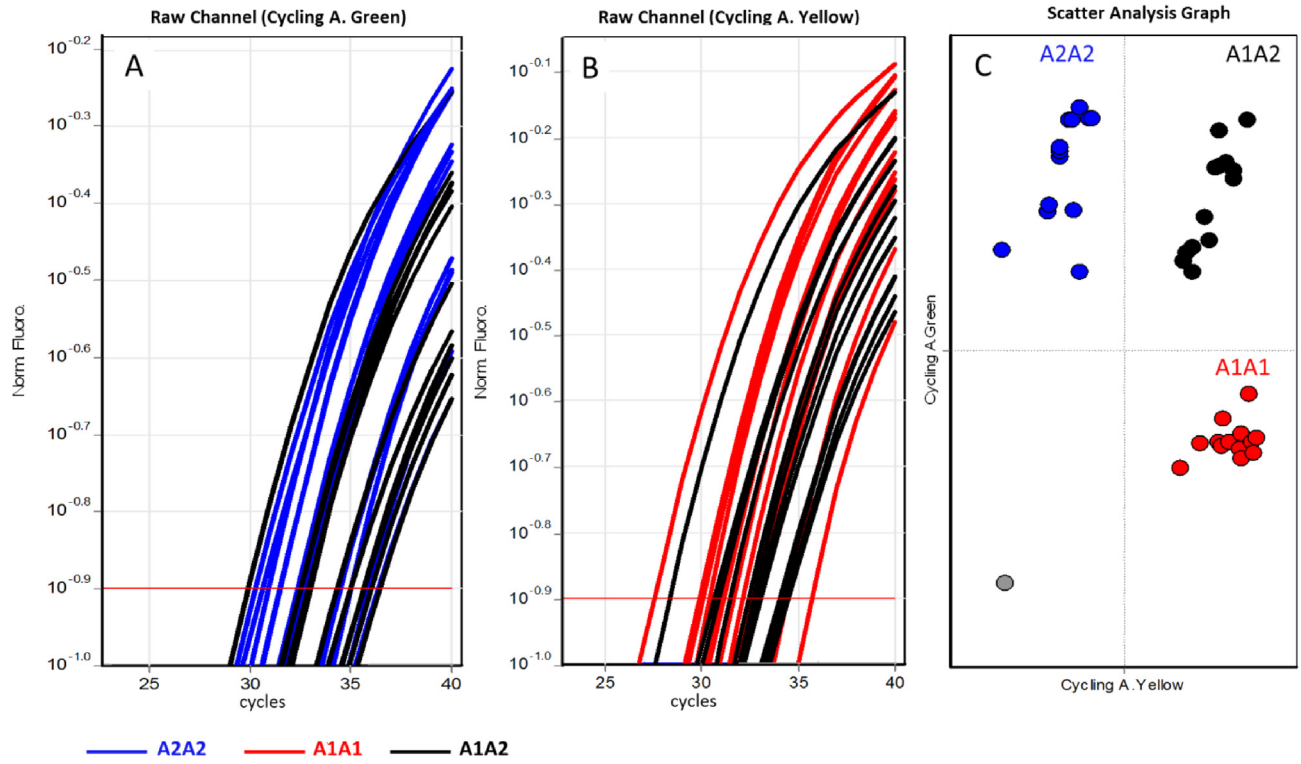

Fig. 4. Amplification curve (A and B) and allelic discrimination plots (C) for SNPs located in *CSN2* gene obtained by rhAmp genotyping assays on 35 milk samples. The biallelic discrimination was achieved through the competitive binding of two allele-specific forward primers, one labeled with FAM dye and the other with Yakima Yellow (YY) dye. Nonspecific amplifications were not observed. In C: Red (A1) and blue (A2) dots represent the homozygous genotypes, the black circles represent heterozygous genotypes and the grey circle on the bottom left of the plot is no-template control. Quantitative cycles are represented on a logarithmic scale (A and B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Results of analytical sensitivity tests for A1 identification in A2 samples by HRM and rhAmp assays using synthetic DNA and sequenced samples. Red letters highlight the A1 concentrations below the limits of detection.

		HRM analysis		rhAmp™
Sample type	% A1 in A2	Genotype	Confidence %*	
Sequenced sample	100	A1A1	99.4	A1A1
	50	A1A2	99.7	A1A2
	40	A1A2	99.2	A1A2
	30	A1A2	99.2	A1A2
	20	A1A2	96.4	A1A2
	10	A1A2	94.4	A1A2
	5	Variation	-	A1A2
	2	A2A2	99.6	A1A2
	1	A2A2	98.9	A2A2
	0 (A2A2)	A2A2	99.4	A2A2
Synthetic DNA	100	A1A1	98.7	A1A1
	50	A1A2	92.9	A1A2
	40	A1A2	95.2	A1A2
	30	A1A2	94.8	A1A2
	20	A1A2	98.1	A1A2
	10	A1A2	97.7	A1A2
	5	A1A2	98.9	A1A2
	2	A2A2	95.6	A1A2
	1	A2A2	96.9	A2A2
	0 (A2A2)	A2A2	98.9	A2A2

*% confidence refers to the means of the duplicates of each sample and for each method.

identification of A1 allele presence in the A2 samples, the HRM analysis showed a limit of detection of 10% (100 copies), whereas for rhAmp the limit was 2% (10 copies). Table 1 presents the results of the genotyping via HRM and rhAmp techniques. Improved efficiencies of A1 detection in the A2 samples were found for both methods. When synthetic DNA was used, the observed limits were 5% (50 copies) and 2% (10 copies) for HRM and rhAmp, respectively (Table 1 and Fig. 5).

4. Discussion

A2 β-casein milk is becoming increasingly popular among consumers and producers. Farmers in many regions of the world are being encouraged to produce A2 milk to meet the demand for this product, which is considered a healthier alternative to conventional dairy (Bell, Grochoski, & Clarke, 2006). Brazil has recently started to produce commercial A2 milk. Currently, A1-free bovine milk is being marketed in several countries, including Australia, the United Kingdom, the United States, New Zealand and the Netherlands (Brooke-Taylor et al., 2017).

This study is the first attempt to develop HRM and rhAmp assays for genotyping *CSN2* alleles in milk samples. Most researchers were focused on identifying the frequencies of these genotypes in the animals (Chessa, Bulgari, Rossoni, Ceriotti, & Caroli, 2013; Dai et al., 2016; Ganguly et al., 2013; Rangel et al., 2017; Royo et al., 2014; Sharma et al., 2013). Although we have also determined the genotypic frequencies, these results were not the main focus of this study. The priority was to develop a rapid and high-sensitive method for A1 allele identification in A2 milk samples. The final concentrations of the DNA extracted from samples of milk (each animal) ranged from 10 to 100 ng/μL (data not shown), which allowed the dilution to 5 ng/μL, which was then used in both genotyping methods. This variation of DNA quantity may be associated with the number of somatic cells present in the milk. Although the somatic cell count in milk is used as an important indicator of udder health, it may also be influenced by several factors, such as animal species, milk production level, lactation stage, and individual and environmental factors, as well as management practices (Li, Richoux, Boutinaud, Martin, & Gagnaire, 2014).

The HRM and rhAmp methods developed facilitated the identification of the two most common variants of bovine β-casein, with considerable accuracy. The sequenced PCR products demonstrated that the

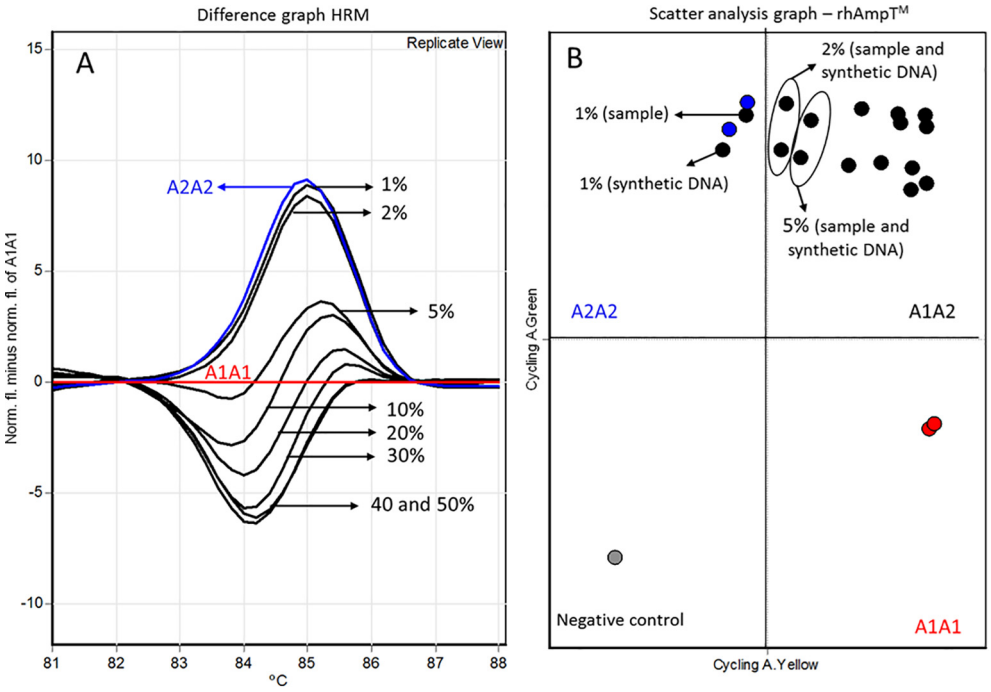

Fig. 5. Analytical sensitivity test for A1 identification in A2 samples for HRM analysis (A) and rhAmp genotyping (B). Synthetic DNA and sequenced DNA samples containing initially the proportion 50% of each allele (500 copies of each allele), the percentages of A1 were progressively decreased: 50% (500 copies), 40% (400 copies), 30% (300 copies), 20% (200 copies), 10% (100 copies), 5% (50 copies), 2% (10 copies) and 1% (5 copies). The values of HRM analysis are showed as replicates means between gBlocks and sequencing samples, while for rhAmp test are separated.

region flanked by primers (HRM) and probes (rhAmp) was highly conserved and contained only the mutation C/A (A2 to A1). The identity between the 10 sequenced samples and genotyped sequences previously deposited in GenBank was 100%, enabling their use as positive controls in subsequent analyses. The synthetic fragments' gBlocks were also used as positive controls and presented the same patterns as the sequenced samples.

In the HRM analysis, changes in the shape of the normalised curves were observed between the different genotypes. The rhAmp genotyping also enabled the three genotypes to be discriminated. Thus, both methods proved to be highly reliable for the determination of the homozygous A1A1 and A2A2 and the heterozygous A1A2 genotypes in samples obtained from both blood and milk. In this study, we used animals that had been genotyped from a milk producer of A2A2 cows. For both techniques, there was 100% agreement with the previous genotyping results of these animals. Therefore, we suggest that both strategies might be used, instead of the DNA sequencing technique.

Given that demand for the consumption of A2 (A2A2) milk is increasing across the world (Gaudry et al., 2019), accurate methods by which to identify the A1 allele in milk commercialized as A2 are also highly valuable. Consequently, identification of animal genotypes may be not completely sufficient. Rather, a method that detects a minimal presence of the A1 allele in milk samples commercialised as A2 could prove to be an excellent tool. The HRM and rhAmp assays used here detected the presence of allele A1 in A2 samples at rates of 10% (100 copies) and 2% (10 copies), respectively. The rhAmp was ten times more sensitive than HRM analysis for this purpose. However, when synthetic DNA was used, the limits of detection were 5% and 2% for HRM and rhAmp, respectively. Thus, we may conclude that both methods are highly sensitive for genotyping animals from milk samples. Nevertheless, we recommend the rhAmp method to detect the contamination of A1 in A2 samples, due to its increased sensitivity.

Literature studies standardising the HRM method for the genotyping of variants A1 and A2 remain scarce. Sharma et al. (2013) genotyped 314 cows from various places in North India by HRM analysis in order to discriminate β-casein A1 from A2, ultimately deeming this method a straightforward strategy to detect and characterise these genotypes. Royo et al. (2014) have also used HRM analysis to identify A1 and A2 genotypes simultaneously, with the method proving to be a good alternative for routine typing of a low or medium number of polymorphisms, as true of the most frequent β-casein variants. The present study has represented the first attempt to standardise the rhAmp method for genotyping β-casein A1 and A2 allelic variants. A similar investigation by Manga and Dvořák (2010) to distinguish between A1 and A2 alleles using hydrolysis probes system (TaqMan), found 100% of genotyping accuracy and a 100-fold greater degree of sensitivity than ACRS-PCR (amplification created restriction site method followed by PCR assay) method.

5. Conclusions

In conclusion, this study has demonstrated that HRM and rhAmp methods reliably discriminate and detect A1A1, A2A2 and A1A2 genotypes of the *CSN2* gene in milk samples. Although both techniques presented 100% accuracy for genotyping animals, the rhAmp demonstrated ten times greater sensitivity than the HRM method in terms of its sensitivity towards the presence of small quantities of A1 in an A2 sample. Thus, it is confidently suggested here that rhAmp could be used to detect small quantities of the A1 allele in A2 samples and hence to identify A1 in milk commercialised as A2.

CRediT authorship contribution statement

Rodrigo Giglioti: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Gunta Gutmanis:** Conceptualization, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Luciana Morita Katiki:** Conceptualization, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Cintia Hiromi Okino:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Márcia Cristina de Sena Oliveira:** Conceptualization, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Aníbal Eugênio Vercesi-Filho:** Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing - original

draft, Writing - review & editing.

Declaration of Competing Interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126167.

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