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Novel LNA probe-based assay for the A1 and A2 identification of β-casein gene in milk samples

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ABSTRACT

The rising consumption of *A2* milk and its derivatives in recent years has garnered attention from both consumers and producers, mainly due its possible health benefits, such as enhanced digestion and easier absorption. Thus, a novel real-time PCR using a combination of locked nucleic acid modified (LNA) conjugated probes was developed to genotype *A1* and *A2* alleles of *β*-casein gene (*CSN2*) and to detect and quantify the *A1* presence in *A2* samples. The limit of detection for each probe (*A1* and *A2*) was evaluated using decreasing serial dilutions. Besides, the sensitivity of A1 allele detection in the *A2* samples was also tested. The limits of detection of *A1* and *A2* alleles were 6 copies, while for *A1* allele detection in *A2* samples was 7.5 copies (1%). The LNA-probe based method was found to be rapid, robust, highly sensitive, cost effective, and can be employed as screening test to certificate the *A2* dairy products.

1. Introduction

β-casein A1 and A2 variants have been recently gaining increasing interest from both researchers and consumers, stimulating a new trend in the dairy market, consequently, producers in many countries of the world have started to produce A2 milk (Mendes et al., 2019; Bentivoglio et al., 2020).

The caseins constitute 80% of bovine milk proteins and have four forms: α_{S1}(*CSN1-S1*), α_{S2}(*CSN1-S2*), *β*-(*CSN2*) and κ-(*CSN3*) in the approximate proportions 4:1:4:1, respectively (Visser et al., 1991). β-casein gene has 13 allelic variants, and the most frequently found in cattle are *A1* and *A2* variants (Farrell et al., 2004). The difference between *A1* and *A2* alleles is a mutation at amino acid 67 (proline in *A2* by histidine in *A1*) (Bonfatti et al., 2010). The histidine (A1 allelic variation) results in the cleavage of the preceding seven amino acid residues, generating the bioactive peptide *β*-casomorphin-7 (*βCM-7*) (Jinsmaa & Yoshikawa, 1999).

The *βCM-7* is yielded by the successive gastrointestinal proteolytic digestion of bovine *β*-casein variants *A1* and *B*, but this was not observed for *A2* variant (Kamiński et al., 2007). De Noni (2008) evaluated the *βCM-7* release during simulated gastro-intestinal digestion of bovine *β*-casein variants and milk-based infant formulas, and verified that this peptide was not released from variant *A2* during whole experimental study. Hohmann et al., (2021) evaluated the serum levels of intact *βCM-7* in calves fed with milk containing *A1* or *A2* *β*-casein, and verified that intact *βCM-7* was 5 times higher detected in *A1*-calves. According to these authors, calves supplemented with *A2*-milk presented minimized cleaved opioid peptide *βCM-7* levels and might have advantages in the development of pre-weaned dairy calves. Haq et al., (2014) and Barnett et al., (2014) concluded that the consumption of milk derivatives containing *A1* variants of *β*-casein induced inflammatory response in mice and rats' gut by activating the *Th2* pathway. Furthermore, consumption of milk containing *A1* *β*-casein was associated with increased gastrointestinal inflammation, worsening of symptoms of post-dairy digestive discomfort, delayed transit, and decreased cognitive processing speed and accuracy (Jianqin et al., 2016).

The genotyping tests of beta-casein *CNS2* gene in cattle herds have increased considerably over the last few years, since the demand for cattle with *A2A2* genotypes has intensified due to the greater demand for milk and dairy products exclusively of *A2* type. However, most of the research is strictly related to genotyping the animals (Gustavsson et al., 2014; Kamiński et al., 2006; Keating et al., 2008; Lien et al., 1999; Rangel et al., 2017; Royo et al., 2014; Visker et al., 2010; Ristanic et al., 2020; Schettini et al., 2020; Ivanković et al., 2021; Ladyka et al., 2021;

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Teixeira et al., 2021). The first study that was developed to genotype the *CSN2* gene alleles directly in milk samples was accomplished by Giglioti et al., (2020), that evaluated two methods: high-resolution melting (HRM) and rhAmp® SNP genotyping. According to the authors both methods were able to discriminate genotypes from *CSN2* gene in milk samples, and the rhAmp method demonstrated ten times higher sensitivity for detection of the presence of *A1* in *A2* milk samples than the HRM method.

Therefore, the present study aimed to develop a new real-time PCR method using a locked nucleic acid (LNA) probe for the detection of the *A1* and *A2* allelic variations in individual cattle samples, and to evaluate the sensitivity for detection of *A1* in *A2* milk samples. LNA is a nucleic acid analog with a 2′-O,4′-C methylene bridge (Koshkin et al., 1998), which enhances the probe performance compared to classical hydrolysis probes, and allows shorter probe designs (Josefsen, et al., 2009). The LNA probes have been used to differentiate variations between the same species with a single base mutation (Ugozzoli et al., 2004) and have been employed in food-safety-based assays (Josefsen, et al., 2009).

2. 2-Material and methods

2.1. Experimental samples

Thirty-eight hair follicles samples were collected from the cow's tail by plucking them from their roots. The cows belong to an experimental farm of the Instituto de Zootecnia, Nova Odessa, São Paulo state, Brazil (22°46′39″ S, 47°17′45″ W; 570 m altitude). The milk DNA samples were obtained from a previous study (Giglioti et al., 2020), that included the samples *A1A1* and *A2A2* identified by Sanger sequencing and synthetic fragments gBlocks® (gene fragments containing *A1* and *A2* alleles). This study was approved by the Ethics Committee on Animal Use of the Instituto de Zootecnia (Protocol Nr. 272/18).

2.2. DNA extraction

DNA extraction from hair follicles 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. The DNA was eluted in 20 μL of Tris-EDTA. Regarding the DNA extraction from milk, the samples were pre-processed according to the method by Reale et al. (2008), following the modifications recommended by Giglioti et al. (2020). The quantification and the purity of the extracted DNA were estimated by spectrophotometric readings at 260 nm and 260/280 nm ratios, respectively. The DNA concentrations of all tested samples were adjusted to 5 ng μL^{-1}.

2.3. qPCR primers and LNA probe design

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). A set of primers were constructed from sequences flanking a fragment of 73 nucleotides located in the bovine *β*-casein encoding gene (*CSN2* gene; sequences n. MK426695.1 and MK426696.1) as follows; sense primer: 5′-ACAGTCTCTAGTCTATCCCTTC-3′; anti-sense primer: 5′-TTGAGTAA-GAGGAGGGATGT-3′. Dual labelled LNA hybridization probes were designed, complementary to the anti-sense DNA strand and spanning the transition site as follows; probe *A1*, 5′-(HEX) agGCtGttATggat (BHQ1)-3′; probe *A2*, 5′-(FAM) ctGTtAGggatGg (BHQ1)-3′. LNA nucleotides are denoted in upper case, DNA nucleotides no locked are denoted in lower case, and the nucleotides complementary to the identified SNP is underlined.

2.4. Real-time PCR optimization

The qPCR assays here developed were optimized by testing optimal conditions of primers, probes, DMSO addition, annealing-extension temperatures. Briefly, each reaction was carried using 5 pmol of primers (0.5 μL of 10 μM each primer solution), 2.5 pmol of each probe (0.5 μL of 2.5 μM probe solution) and 5 × HOT FIREPol Probe Universal qPCR Mix (2 μL of 5 × Mastermix solution) (Solis BioDyne, Tartu, Estonia), 4 μL of ultrapure water and 2 μL of DNA in Rotor-Gene Q thermocycler (Qiagen, Venlo, Netherlands). Optimal thermal profiles were set at 95 °C for 10 min, 45 cycles of 95 °C (denaturation) for 15 s and 65 °C (annealing/extension) for 60 s.

2.5. Analytical sensitivity

The analytical sensitivity was evaluated using two different tests: (i) heterozygous sample (*A1A2*) containing 750 DNA copies were submitted to serial 5-fold dilutions (750, 150, 30, 6, and 1.2 copies), using eight technical replicates and were submitted to qPCR runs; (ii) sensitivity of A1 allele detection in *A2* sample: were evaluated decreasing concentrations of *A1* in *A2* sample: 90% (675 copies), 80% (600 copies), 70% (525 copies), 60% (450 copies), 50% (375 copies), 40% (300 copies), 30% (225 copies), 20% (150 copies), 10% (75 copies), 5% (37,5 copies), 2% (15 copies), 1% (7.5 copies), and 0.5% (3.75 copies). The tests were evaluated using synthetic DNA (*A1* and *A2*) and DNA from milk samples (*A2A2*, *A1A1* and *A1A2*) as controls in each assay (Giglioti et al., 2020). The limit of detection was set at last dilution which presented $\geq$ 90% of detection in 10 independent qPCR run.

2.6. Comparative test

The LNA-probe method accuracy was compared with rhAmp assay following the methodology described by Giglioti et al. (2020), in which the results from 38 cattle DNA samples were compared between two methods. The quantitative cycles (Cq) from each channel (yellow-*A1*, and Green-*A2*) were submitted to analysis of variance using GLM of SAS (SAS Institute, Cary, NC, US) and the means were compared according to each channel and qPCR method by Tukey's test at 5% of significance.

3. Results

The LNA-probe assay successfully genotyped all DNA samples (Fig. 1). The *A1A1*, *A1A2* and *A2A2* genotype frequencies of evaluated animals were 13.5%, 48.6% and 37.8%, respectively (Fig. 1). Nonspecific amplifications were not observed.

The comparison between the results provided by LNA-probe and rhAmp showed 100% of agreement. The Cq means obtained from yellow and green channels and for LNA-probe method were 27.46 ± 1.19 and 27.86 ± 1.93, while for rhAmp assay were 30.84 ± 0.91, and 32.44 ± 1.43, respectively (Fig. 2. The Cq means from the yellow and green channels from rhAmp method differed significantly from each other ($P < 0.05$), and were also significantly higher than Cq means from LNA-probe assay. The Cqs means between the two channels from LNA-probe presented no significant difference ($P > 0.05$).

The limit of detection found at test "i" was 6 copies, and Cq means observed for yellow and green channels were 32.9 ± 0.5 and 35.6 ± 1.9, respectively (Fig. 3 A and B). The efficiency rate observed in the qPCR amplification curves at yellow and green channels were 96% and 95%, respectively. The limit of detection of *A1* allele in the *A2* samples was 1% (7.5 DNA copies) (Fig. 4 A and B).

4. Discussion

The global demand for milk production from cows containing only the *A2A2* genotype has increased in recent years. Consequently, animal genotyping tests to select animals containing only the *A2* allele and the

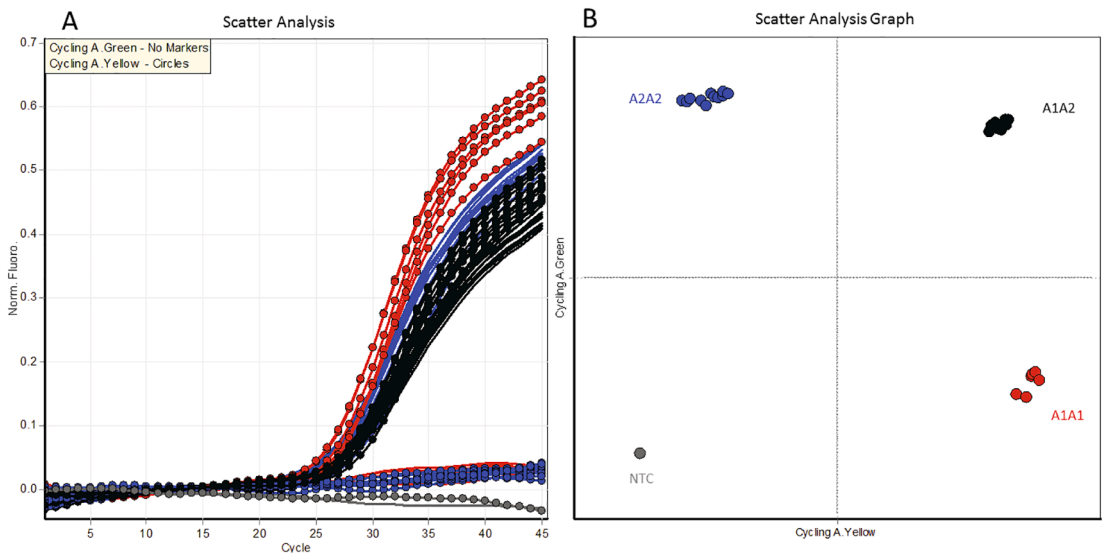

Fig. 1. Amplification curves (A) and allelic discrimination plots (B) obtained by LNA-probe genotyping assays on cattle DNA samples (n = 20). A) Straight line - *A2* sample detection (green channel - FAM), lines with circles - A1 detection (yellow channel - HEX); B) Red (A1) and blue (A2) dots represents the homozygous genotypes, the black circles represents heterozygous genotypes and the grey circle on the bottom left of the plot is the no-template control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

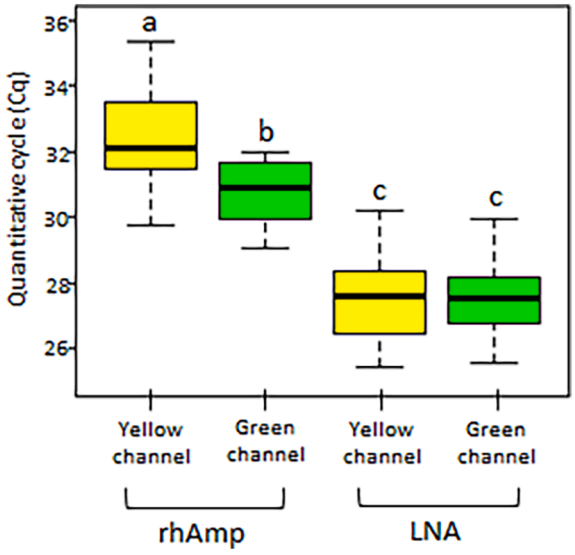

Fig. 2. Quantitative cycle (Cq) means obtained from yellow and green channels by LNA probe and rhAMP qPCR assays using 38 DNA samples. Means followed by the different letters differ significantly ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

detection of the presence of the *A1* allele in dairy products are highly required. In this context, there are several methodologies developed for *CNS2* gene of β-casein *A1* and *A2* alleles genotyping (Chessa et al., 2013; Dai et al., 2016; Ganguly et al., 2013; Rangel et al., 2017; Royo et al., 2014; Sharma et al., 2013; Giglioti et al., 2020), but the present study was the first one to incorporate a real-time PCR using LNA probes.

The use of PCR using LNA-probe in food products has been extensively applied, such as the detection of *Trichinella* in meat (Alonso et al., 2011), detection and identification (authentication) of Atlantic salmon (Herrero et al., 2011), detection of cashew nut in processed food (Sanchiz et al., 2018), and detection of allergenic walnut in complex food matrices (Puente-Lelievre and Eischeid, 2021). All of these studies mentioned above showed that the use of the LNA-probe system have improved the sensitivity, specificity and applicability for the detection of the same target compared to other Real Time PCR methods using other systems of detection. In our study, the LNA-probe method reliably discriminated and detected *A1A1*, *A2A2* and *A1A2* genotypes of the *CSN2* gene in cattle DNA samples. Josefsen et al., (2009) evaluated real-time PCR assays for detection of food-borne thermotolerant *Campylobacter* and verified that the LNA probe produced significantly lower Cq-values and a higher proportion of positive qPCR results than the hydrolysis probe system. In addition, these authors verified that the MGB (minor groove binder) probe was not found to be superior to the hydrolysis probe system.

The present study was partially based on the previous study proposed by Giglioti et al., (2020) which developed two methods – high-resolution melting (HRM) and rhAmp® SNP genotyping – to identify *A1* and *A2* alleles directly in milk. The high accuracy for genotyping and detection of *A1* and *A2* alleles using LNA-probe in this study were similar to those found by these authors. Regarding the comparison between LNA-probe assay and rhAmp method (Giglioti et al., 2020), the results showed 100% of agreement. However, the Cq means from yellow and green channels obtained from LNA-probe assay were significantly lower compared to those obtained by the rhAmp assay. The Cq mean differences between the LNA-probe and rhAmp assays for yellow and green channels were 4.6 and 3.4, respectively. Reynisson et al., (2006) and Josefsen et al., (2009) verified an improved performance of LNA probes compared with a TaqMan probes, wherein the LNA-probe produced lower Cq-values and standard deviations. Although the sensitivity found in the LNA-probe system in the present study was similar to that found in the rhAmp system (Giglioti et al., 2020), the results found in the comparative study for the genotyping of cattle samples, the LNA-probe method showed Cq-values significantly lower when compared to the rhAmp system. Thus, we can suggest that the use of the LNA-probe method may improve the detection performance to genotyping cattle DNA samples for detecting the *A1* and *A2* alleles. In addition, the Cq means obtained from the two different LNA-probes presented no differences. In contrast, for rhAmp assay the yellow channel (A1*)* presented greater detection than the green channel (*A2*).

In the present study, we evaluated two different procedures to assess detection sensitivity ("i" and "ii"), as we hypothesized that increasing the concentration of the *A2* allele (and consequently the increase in *A2* amplicons) could decrease the sensitivity of detection of the *A1* allele in

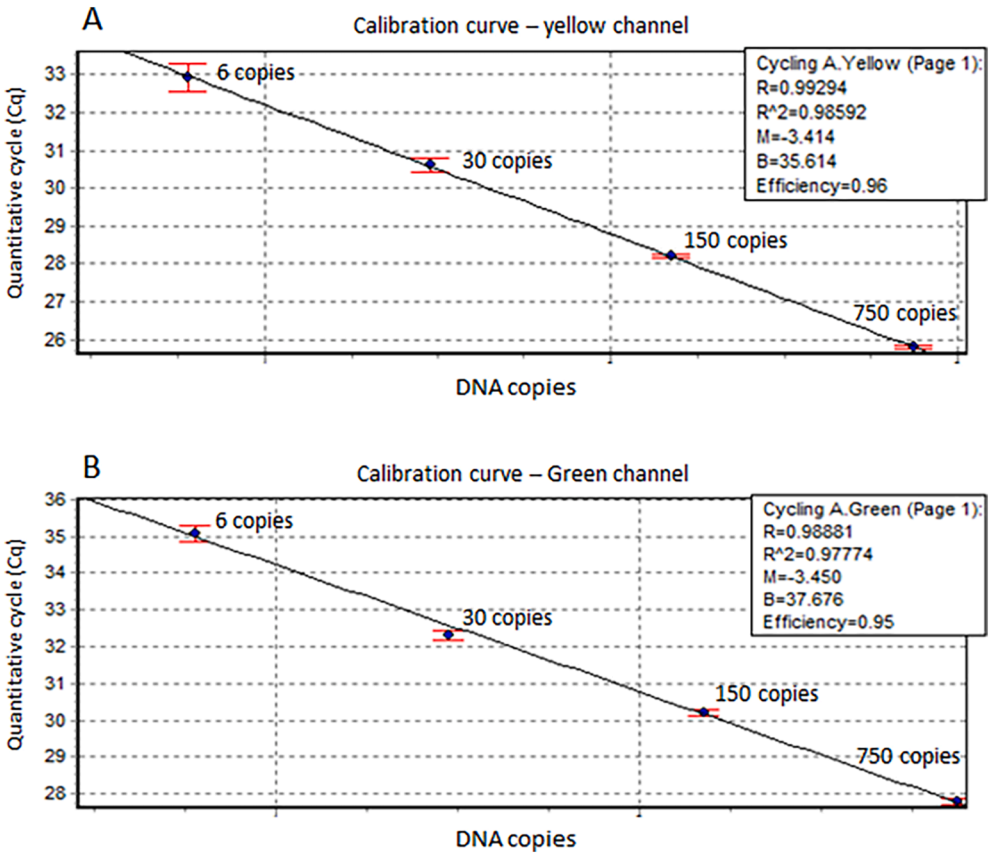

Fig. 3. Linear regressions obtained by plotting Cq mean values and respective 5-fold serial dilutions of synthetic DNA (A1 and A2) obtained in analytical test "i", including the results of correlation coefficient, efficiency and y-intercept.

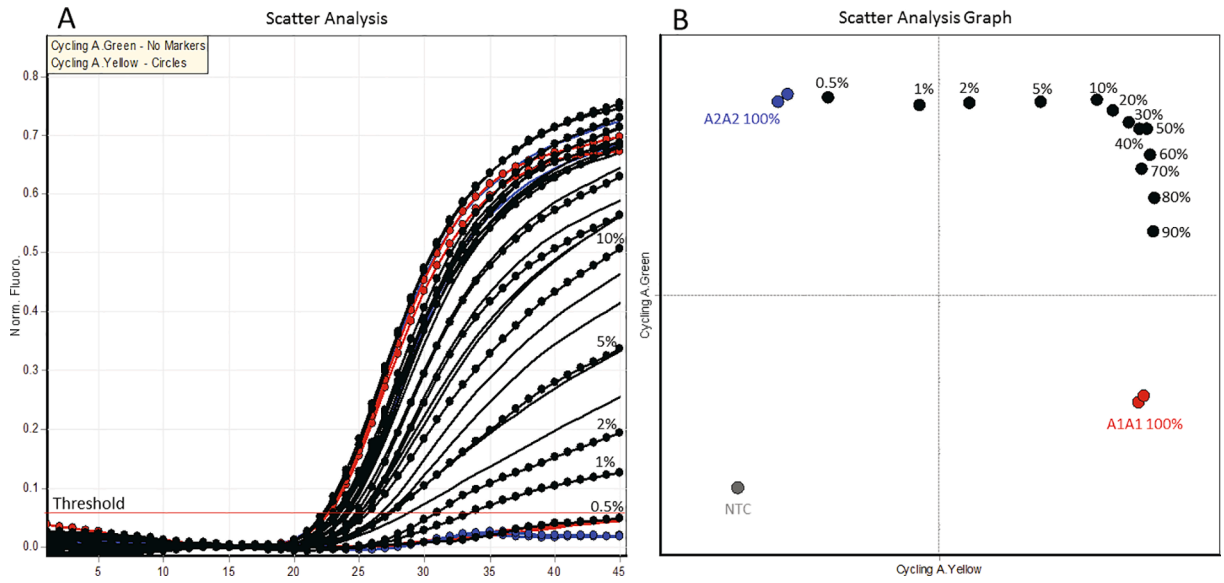

Fig. 4. Results obtained from analytical sensitivity test "ii", for A1 identification in A2 samples obtained by LNA-probe assay. A- Amplification from allelic discrimination analysis; Straight line - *A2* sample detection (yellow channel) and solid lines - *A2* detection (green channel). B- Scatter analysis graph: red (*A1*) and blue (*A2*) dots represents the homozygous genotypes, the black circles represents the percentages of *A1* in *A2*, and the grey circle on the bottom left of the plot is no-template control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A2 samples. The limit of detection observed in the analytical test "i" was 6 copies, while in the test "ii", the detection of *A1* in *A2* samples was 7.5 copies (1%). Based on these results, we concluded that the LNA-probe system developed was able to detect *A1* allele in very low concentrations, similarly to rhAmp method (Giglioti et al., 2020), and can be used to detect the presence of the *A1* allele in *A2* samples.

Although the LNA-probe and rhAmp methods presented similar analytical sensitivity, we can point out two advantages of the LNA-probe method compared to the use of the rhAmp method: (i) several companies can commercialize and / or synthesize LNA probes or primers, while

rhAmp only one company can commercialize due to patent holding; (ii) based on the conditions of the present study, the cost evaluated for the LNA-probe method was approximately seven times lower (including all reagents) compared to the rhAmp method.

5. Conclusions

The present study was the first to develop a real-time PCR using LNA-probe for identification of *A1* and *A2* alleles of the *CNS2* gene of *β*-casein directly in milk or hair follicle samples from cattle. This method presented 100% accuracy for genotyping animals and the limits of detection for *A1* and *A2* alleles were 6 copies, while the limit of detection of the *A1* allele in *A2* samples was 1% (7.5 copies). The LNA-probe method was found to be a rapid, robust, highly sensitive, cost effective, may constitute an interesting alternative to rhAmp assay, and can be employed as molecular screening method for dairy producers.

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. **Cintia Hiromi Okino:** Conceptualization, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Bianca Tainá Azevedo:** . **Gunta Gutmanis:** Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Luciana Morita Katiki:** Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Márcia Cristina de Sena Oliveira:** Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Anibal Eugênio Vercesi Filho:** Investigation, Methodology, Project administration, 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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