β-casein gene polymorphism permits identification of bovine milk mixed with bubaline milk in mozzarella cheese

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Abstract

Mozzarella cheese is traditionally prepared from bubaline (Bubalus bubalis) milk, but product adulteration occurs mainly by addition of or full substitution by bovine milk. The aim of this study was to show the usefulness of molecular markers to identify the admixture of bovine milk to bubaline milk during the manufacturing process of mozzarella cheese. Samples of mozzarella cheese were produced by adding seven different concentrations of bovine milk: 0%, 1%, 2%, 5%, 8%, 12% and 100%. DNA extracted from somatic cells found in cheese were submitted to PCR-RFLP analysis of casein genes: α-s1-CN – CSN1S1 that encompasses 954 bp from exon VII to intron IX (Alu I and Hinf I), β-CN – CSN2 including 495 bp of exon VII (Hae III and Hinf I), and κ-CN – CSN3, encompassing 373 bp of exon IV (Alu I and Hind III). Our results indicate that Hae III-RFLP of CSN2 exon VII can be used as a molecular marker to detect the presence of bovine milk in “mozzarella” cheese.

Key words: casein, gene polymorphism, mozzarella, cheese authentication, PCR-RFLP.

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Mozzarella cheese has its origin in Italy where it is traditionally manufactured from bubaline (Bubalis bubalis) milk. The most commonly employed production process of mozzarella cheese is a traditional technique where bacterial fermentation of milk induces pH reduction and curd precipitation (Chapman et al., 1981). Nowadays, many countries that have a considerable number of buffalo cows widely use the milk of these animals for mozzarella cheese production.

Some dairy products can be adulterated by milk admixture from different species in order to maximize profit. Certification is, thus, a way to guarantee cheese quality and to protect consumers against fraudulent producers. Bubaline breeders demand high precision technology to validate milk origin to assure that only bubaline milk is present in the composition of the manufactured product. The most common type of adulteration in the manufacture of mozzarella cheese is the addition or full replacement of bubaline milk by bovine milk. Consequently, several methods have been developed to detect milk mixture in these products. Methods based on electrophoresis and chromatography include isoelectric focusing (Moio et al., 1989), high-performance liquid chromatography (Visser et al., 1991; Veloso et al., 2002; Enne et al., 2005), nuclear magnetic resonance spectroscopy (Andriotti et al., 2000), and also hydrophobic interaction chromatography (Bramanti et al., 2003). However, these methods present limitations due to time intensive protocols and/or high costs.

An alternative way to detect milk mixtures is the use of molecular markers to identify the DNA of different species (Bardin et al. 1994; Branciari et al., 2000; Rea et al., 2001; Bottero et al., 2002; Leoparelli et al., 2007). Here we describe a relatively rapid and simple method to identify admixtures of bovine milk to bubaline milk, by extracting DNA directly from Mozzarella cheese and analyzing a β-casein gene polymorphism.

Samples of “pasta filata” mozzarella cheese were produced using 7 L of milk according to the methodology developed by Kuo et al. (2001). The samples were prepared in the “Laticínio White Milk” facility (Marilia, SP, Brazil), using bovine milk mixed with bubaline milk at concentrations of 0%, 1%, 2%, 5%, 8%, 12% and 100% (T1-T7). Bovine milk was obtained from a herd of different milky breeds (Holstein, Brown-Swiss, Jersey, and the Brazilian native breeds: Gir, Guzerá and Girolanda) with the intention to have as many β-casein gene variants as possible.

Genomic DNA was extracted from 30 samples of each bovine milk concentration, following a methodology...
adapted by the authors. Each sample consisted of 25 mg mozzarella cheese, 200 µL Nonidet P40 (12.5%), 1 mL TKM1 buffer [Tris-HCl (1 M) pH 7.6; KCl (1 M) and EDTA (0.1 M) pH 8.0] and 200 µL 20% SDS. After heating the sample in a water bath at 55 °C for 1 h, 400 µL NaCl (6 M) were added, followed by centrifugation at 14,840 xg for 15 min at 31 °C. A 700 µL aliquot of the precipitate was transferred to a new tube, and 700 µL of cooled absolute ethanol were added, followed by centrifugation at 14,840 xg for 15 min at 4 °C. The residual solution was then discarded, and 100 µL of TE buffer [Tris-HCl (1 M) pH 8.0 EDTA (0.1 M) pH 8.0] 10:1 were added.

DNA amplification was performed using primers for the casein genes: α-s1-CN – CSN1S1 (Α900 F 5'-ATGT TGGGCACCTACTGAC-3' and ΑI8 R 5'-GGATAGA GCTACATACATAGT-3') that amplifies a 954 bp fragment from exon VII to intron IX; β-CN – CSN2 (ΒF 5'-GATGAACTCCAGGATAAAATC-3' and ΒR 5'-ATAATAGGGAAGGGTCCCCG-3') that amplifies a 495 bp fragment of exon VII; and κ-CN – CSN3 (K1 5'-CACGTCACCCACACCCACATTTATC-3' and K2 5'-TAATTAGCCATTTCGCCTTCTCTGT-3') that amplifies a 373 bp fragment of exon IV. PCR amplifications were performed in a final volume of 25 µL containing 100 ng of DNA, 0.5 µM of each primer, 1 X PCR “buffer” (10 mM Tris-HCl pH 9.0, 1.5 mM MgCl2 and 50 mM KCl), 100 µM of dNTPs, 0.5 U of Platinum Taq DNA polymerase (Invitrogen). The amplifying cycles followed the steps: 95 °C for 5 min, 30 cycles (95 °C for 30 s; 59 °C for 60 s; 72 °C for 60 s) and 72 °C for 5 min. Amplified fragments were checked by electrophoresis (40 min at 80V) of 5 µL of each reaction product on agarose gels (1.5%) containing ethidium bromide. Fragments were visualized under UV light using a Gel Logic system (Kodak).

For restriction fragment length polymorphism (RFLP) analysis, 5 µL of each amplified sample was digested at 37 °C for 1 h with the appropriate restriction enzymes, following manufacturer instructions (Invitrogen). A 15 µL aliquot of each digested sample was electrophoresed (90 min at 80V) on a 3% agarose gel prepared with TEB 1X (Tris-HCl 89 mM; EDTA 2.5 mM; boric acid 89 mM; pH 8.3), and ethidium bromide (5.0 µg/mL). Alternatively, electrophoresis was performed on an acrylamide:bis-acrylamide (49:1) 12% non-denaturing gel, using a SequiGen 38x50 apparatus (BioRad®). Runs were performed for 60 min at 80 V followed by staining with ethidium bromide (5.0 µg/mL). The DNA fragments were visualized as described above.

RFLP analysis was based on our previous study of CSN1S1, CSN2, CSN3 genes in a total of 200 lactating female buffaloes and 360 cows, 60 of each of six breeds (Holstein, Brown-Swiss, Jersey, and three Brazilian native breeds, Gir, Guzerá and Girolanda). Nucleotide sequences had been deposited in GenBank by Otaviano and co-workers, and received the following access numbers: CSN1S1 Bubalus bubalis – EF133464, Bos taurus – EF138810 and Bos taurus indicus – EF138811; CSN2 Bubalus bubalis – EF115306, Bos taurus – EF123100 and Bos taurus indicus – EF133460; CSN3 - Bubalus bubalis – EF133463, Bos taurus – EF133462 and Bos taurus indicus – EF133461. These sequences were used to select appropriate restriction enzymes. Only the bovine variants CSN2 Bos taurus –EF123100 and CSN2 Bos taurus indicus – EF133460 were predicted to differ from the bubaline variant CSN2 Bubalus bubalis – EF115306, by a Hae III polymorphism.

As predicted, only CSN2 Hae III-RFLP distinguished between the bovine and bubaline casein genes after electrophoresis on a 3% agarose gel (Figure 1). The intensity of the bands allowed the identification of even very low concentrations of bovine milk added in the process of manufacturing mozzarella cheese. After electrophoresis on the

![Figure 1](image-url) - Hae III-RFLP of CSN2 exon VII allows identifying bovine milk added to bubaline milk in mozzarella cheese. Lane 1, undigested 495 bp PCR product; lanes 2 to 20, bubaline (436 bp) and bovine (373 bp) Hae III-RFLPs obtained from mozzarella cheese containing 0 to 100% bovine milk; concentrations of bovine milk: 0% (lanes 2 and 3), 1% (lanes 4 to 6), 2% (lanes 7 to 9), 5% (lanes 10 to 12), 8% (lanes 13 to 15), 12% (lanes 16 to 18), and 100% (lanes 19 and 20). M, 1 kb Plus DNA Ladder. Restriction fragments less than 80 bp cannot be visualized on this 3% agarose gel (see Figure 2).
acrylamide gel system, the bovine and bubaline CSN2 Hae III-RFLPs products could be distinguished, not only by the larger 373 bp and 436 bp fragments but also by the small 59 bp and 63 bp fragments (Figure 2).

We also considered other bovine CSN2 gene variants (described by Bonsing et al., 1988; GenBank accession number X14711) and bubaline CSN2 gene variants (Singh and co-workers, GenBank; accession numbers: A-DQ191172.1, B-DQ191171.1 and C-DQ191170.1) and performed virtual analyses using pDRAW32 software (Figure 3). The bubaline variants showed 95% sequence similarity with the bubaline variant described here, but they could be distinguished from the bovine variant by the smaller Hae III fragments.

The other RFLPs studied, CSN1S1 (Alu I and Hinf I), CSN2 (Hinf I) and CSN3 (Alu I, Hind III) did not show any difference among bovine and bubaline fragments. The CSN1S1 954 bp fragment resulting from digestion with Alu I generated fragments of 651 and 303 bp, and the one resulting from digestion with Hinf I generated fragments of 298, 248, 213 and 195 bp at all concentrations of bovine milk (experiments T1 to T7). The same occurred with the 373 bp fragment of CSN3 digested by Alu I, which generated fragments of 222, 139 and 12 bp, and by the enzyme Hind III, which generated fragments of 224 and 149 bp. The CSN2 495 bp fragment, digested by Hinf I also yielded fragments of 249, 152 and 94 bp in all samples, at the seven bovine milk concentrations. These results confirmed our previous data, showing that bovine and bubaline genes cannot be differentiated by these RFLPs.

Various methods based on DNA analysis have been previously described for identifying bovine milk addition to pure buffalo cheese products. Branciari et al. (2000) reported a PCR-RFLP method on DNA isolated from Italian mozzarella by sequential organic extraction and resin purification. Rea (2001) proposed a duplex-PCR technique to identify bovine and water buffalo DNA in a single PCR assay of milk and mozzarella cheese. Bottero et al. (2002) also developed a duplex-PCR assay and distinguished between fragments of bovine and bubaline mitochondrial cytochrome b genes. More recently, Loparelli et al. (2007) reported a real-time PCR method capable of quantifying bovine milk addition, based on analysis of the bovine mitochondrial cytochrome b gene and the nuclear growth hormone gene.

In this report we show that the Hae III-RFLP pattern of CSN2 exon VII is capable of distinguishing the bubaline and bovine genes, providing a simple method for detecting the presence of bovine milk in mozzarella cheese.

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References


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