Short Communication

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

Antonio R. Otaviano, André Luis F. Lima, Monyka M.M. Laureano, Janete A.D. Sena,
Lúcia G. de Albuquerque and Humberto Tonhati

Departamento de Zootecnia, Faculdade de Ciências Agrárias e Veterinárias,
Universidade Estadual de São Paulo "Júlio de Mesquita Filho", Jaboticabal, SP, Brazil.

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 usefulnes 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, mozarella, cheese authentication, PCR-RFLP.

Received: August 6, 2007; Accepted: July 21, 2008.

Mozzarella cheese has its origin in Italy where it is traditionally manufactured from bubaline (*Bubalus 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.

Send correspondence to Antonio Roberto Otaviano. Departamento de Zootecnia, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual de São Paulo "Júlio de Mesquita Filho", Jaboticabal, Via de Acesso Prof. Paulo Donato Castellane, s/n, Prédio II, 14884-900 Jaboticabal, SP, Brazil. E-mail: aroctaviano@yahoo.com.br.

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* (A 900 F 5'-ATGT TGGGCACCTACTGAC-3' and A I8 R 5'-GGATAGA GCTACATACATAGT-3') that amplifies a 954 bp fragment from exon VII to intron IX; β-CN – *CSN2* (B F 5'-GATGAACTCCAGGATAAAATC-3' and B R 5'-A 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 MgCl$_2$ 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. Nevertheless, we also analyzed the *CSN1* and *CNS3* polymorphisms.

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 con-
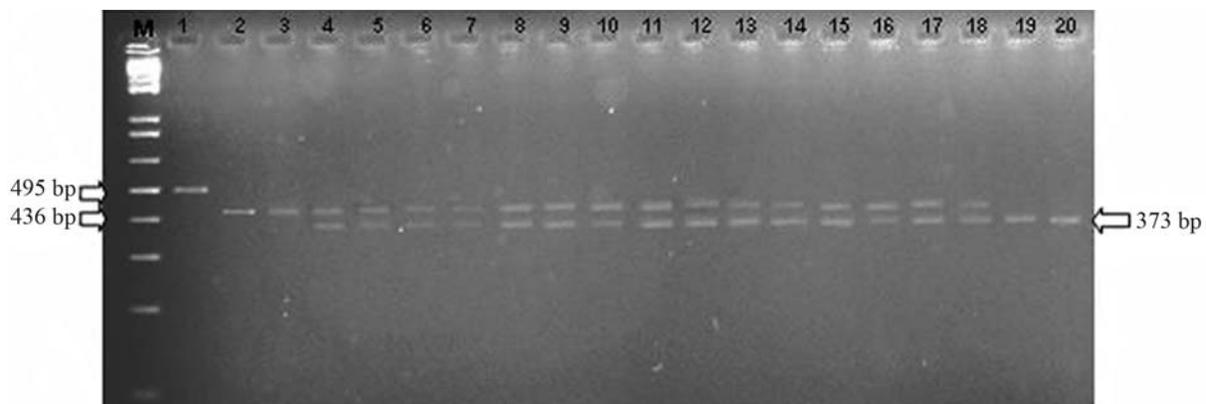

Figure 1 - *Hae* III-RFLP of *CSN2* exon VII allows identifying bovine milk added to bubaline milk in mozzarella cheese. Lane 1, undigested 495 bp PCR product; lanes 2 to 20, bubaline (436 bp) and bovine (373 bp) Hae III-RFLPs obtained from mozzarella cheese containing 0 to 100% bovine milk; concentrations 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).

centrations of bovine milk added in the process of manufacturing mozzarella cheese. After electrophoresis on the 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
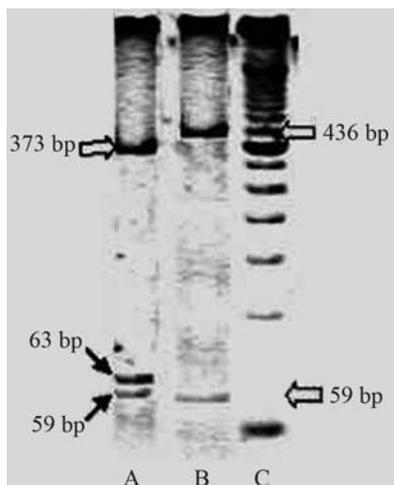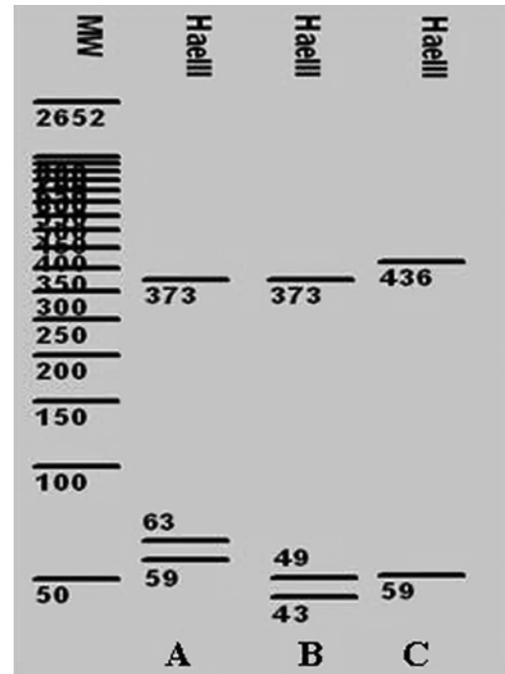

Figure 3 - Virtual analysis of *Hae* III-RFLP products of the *CSN2* exon VII, using the software pDRAW32 on the sequences of (A) bovine and (B) bubaline variants obtained from GenBank; (C) bubaline restriction pattern obtained in this study.

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.

Acknowledgments

Figure 2 - Bovine and bubaline *Hae* III-RFLP of *CSN2* exon VII visualized after electrophoresis on a 12% acrylamide gel: A, 373 bp, 59 bp and 63 bp fragments from cheese prepared with pure bovine milk; B, 436 bp and 59 bp fragments from cheese containing pure bubaline milk; C: 50 bp DNA Ladder.

References

Bonsing J, Ring JM, Stewart AF and Mackinlay AG (1988) Complete nucleotide sequence of the bovine beta-casein gene. Austral J Biol Sci 41:527-537.

Bottero MT, Civera T, Anastasio A, Turi R M and Rosati S (2002) Identification of cow's milk in "buffalo" cheese by duplex polymerase chain reaction. J Food Prot 65:362-366.

Bramanti E, Sortino C, Onor M, Beni F and Raspi G (2003) Separation and determination of denatured alpha (s1)-, alpha (s2)-, beta- and kappa-caseins by hydrophobic interaction chromatography in cows', ewes', and goats' milk, milk mixtures and cheeses. J Chromatogr A 994:59-74.

Branciari R, Nijman IJ, Plas ME, Di Antonio E and Lenstra JA (2000) Species origin of milk in Italian mozzarella and Greek feta cheese. J Food Prot 63:408-411.

Chapman HR and Sharpe ME (1981) Microbiology of cheese. Dairy Microb Appl Sci. Publ 12:157-243.

Enne G, Elez D, Fondrini F, Bonizzi I, Feligini M and Oleandri R (2005) High-performance liquid chromatography of governing liquid to detect illegal bovine milk's addition in water buffalo mozzarella: Comparison with results from raw milk and cheese matrix. J Chromatogr A 1094:169-174.

Kuo MI, Gunasekaran S, Johnson M and Chen C (2001) Nuclear magnetic resonance study of water mobility in pasta filata and non-pasta filata Mozzarella. J Dairy Sci 84:1950-1955.

Lopparelli RM, Cardazzo B, Balzan S, Giaccone V and Novelli E (2007) Real-time TaqMan polymerase chain reaction detection and quantification of cow DNA in pure water buffalo mozzarella cheese: Method validation and its application on commercial samples. J Agric Food Chem 55:3429-3434.

Moio L, Di Luccia A and Addeo F (1989). Fast isoelectric focusing of milk proteins on small ultrathin polyacrylamide gels containing urea. Electrophoresis 10:533-535.

Rea S, Chikuni K, Branciari R, Sangamayya RS, Ranucci D and Avellini P (2001) Use of duplex polymerase chain reaction (duplex-PCR) technique to identify bovine and water-buffalo milk used in making mozzarella cheese. J Dairy Res 68:689-698.

Veloso ACA, Teixeira N and Ferreira I (2002) Separation and quantification of the major casein fractions by reversed-phase high-performance liquid chromatography and urea-polyacrylamide gel electrophoresis detection of milk adulteration. J Chromatogr A 967:209-218.

Visser S, Slangen CJ and Rollema HS (1991) Phenotyping of bovine milk proteins by reversed-phase high-performance liquid chromatography. J Chromatogr 548:361-70.

Internet Resources

pDRAW32 Software, www.acaclone.com.br.
GenBank, www.ncbi.nlm.nih.gov.

Associate Editor: Pedro Franklin Barbosa