UDC 637.352:577.213.38
ISSN 1330-9862
(FTB-1356)

scientific note

Detection of Adulteration in Italian Mozzarella Cheese Using Mitochondrial DNA Templates as Biomarkers

Maria Feligini[1*], *Ivan Bonizzi*[1], *Vlatka Cubric Curik*[2], *Pietro Parma*[1], *Gian Franco Greppi*[1] *and Giuseppe Enne*[1]

[1]Istituto Sperimentale Italiano »Lazzaro Spallanzani«, Viale Papa Giovanni XXIII 7, I-26900 Lodi, Italy

[2]Dairy Science Department, Faculty of Agriculture, University of Zagreb, Svetosimunska 25, HR-10 000 Zagreb, Croatia

Received: July 23, 2004
Revised version: November 4, 2004
Accepted: February 28, 2005

Summary

Considering the importance of monitoring adulterations of genuine cheeses in the dairy industry, a polymerase chain reaction–based method was developed to detect bovine-specific mitochondrial DNA sequence in Italian water buffalo Mozzarella cheese. DNA was isolated from cheese matrix and governing liquid by organic extractions and kit purifications. Amplifications of a 134-bp fragment were performed with a bovine–specific set of primers designed on the sequence alignment of bovine and buffalo mitochondrial cytochrome oxidase subunit I. The specificity of the primers was tested using DNA from the blood of two species (water buffalo and bovine), which are present together in adulterated Italian Mozzarella cheese. This method reliably detected a content of 0.5 % of bovine milk, making it suitable for routine fraud monitoring.

Key words: Italian Mozzarella cheese, mitochondrial DNA, adulteration

Introduction

Fraudulent addition of bovine milk during the manufacturing of water buffalo Mozzarella cheese has increased in recent years, due to the growing market demands in EU (European Union). Several analytical methods have been developed in order to protect consumers and producing countries from fraud originated by the addition of bovine milk in water buffalo Mozzarella cheese. Methods based on protein analysis by chromatographic (*1*), electrophoretic (*2,3*) and immunological (*4*) techniques have been optimized and recently bovine milk has also been detected in water buffalo Mozzarella cheese by mass spectrometry (*5*). All techniques were based on strategies suited to evaluate the protein patterns originating from the major whey proteins or casein fraction. All these analytical methods are able to detect bovine milk proteins in water buffalo Mozzarella cheese to the minimum level of 0.5–1 %.

Identification of animal species using DNA analysis has become more and more effective to detect adulterations in commercial dairy products (*6,7*). DNA from somatic milk cells, principally represented by leucocytes (*8*), persists in cheese and may be analysed for species discrimination. Several PCR-based techniques (DNA hybridization assay; restriction enzyme analysis, RFLP; single-stranded conformation polymorphism analysis, SSCP; duplex polymerase chain reaction, duplex-PCR)

* Corresponding author; Phone: ++39 0371 351 18; Fax: ++39 0371 437 070; E-mail: maria.feligini@isils.it

were performed to amplify nuclear genome obtained from milk and ripened cheese (*9–11*). An adapted DNA extraction procedure and an estimation of the DNA quality, carried out by amplifying a large 3622-bp β-casein sequence (*10*), confirmed that DNA is not completely degraded after cheese manufacturing processes.

Specific polymerase chain reaction (PCR) amplifying a fragment of the cytochrome b gene has been developed for discrimination of species in food ingredients. DNA targets in the mitochondrial genome (*12*) have several advantages over nuclear ones; they are generally more abundant in any given sample than single-copy nuclear genes and contain a greater number of point mutations which can be used for better definition of species differences. By using appropriate primer pairs, mitochondrial sequences were amplified in many species (*13–18*) and the resulting differences were used for species authentication.

Procedures based on mitochondrial cytochrome b gene amplification were also described in order to discriminate bovine milk in buffalo cheese. DNA was recovered from cheese and analyzed by RFLP-PCR (*19*) and duplex PCR (*20*). These methods currently represent valid complements to protein electrophoretic and immunochemical analyses.

On the basis of various studies demonstrating that DNA is not completely degraded after thermal and enzymatic processes involved in Mozzarella cheese production, we propose a new strategy for the detection of low amounts of bovine milk in water buffalo Mozzarella cheese by means of polymerase chain reaction. Our method is based on the amplification of a 134-bp stretch of mitochondrial cytochrome oxidase subunit I gene isolated from cheese matrix and, for the first time, from governing liquid of Mozzarella cheese.

Materials and Methods

Samples

Three experimental lots of Mozzarella cheese were manufactured mixing water buffalo and bovine milk in ratios of 70:30, 80:20, 90:10, 95:5, 99:1 and 99.5:0.5. The cheese and governing liquid samples were preserved at 4–6 °C. Six hours, one week and two weeks after the cheese-making, each lot was frozen at –20 °C to interrupt enzymatic processes. The lots of Mozzarella cheese were made in dairy by traditional manufacturing.

Ten Protected Designation of Origin (PDO) water buffalo Mozzarella cheeses made by several producers in Campania region were purchased in Italian supermarkets and were subjected to two different DNA extraction techniques.

DNA extraction from governing liquid

DNA was isolated from the mixes (six hours, one week and two weeks after the cheese-making), commercial samples, bovine and water buffalo blood by a phenol/chloroform method, followed by ethanol precipitation. Aliquots (40 mL) of governing liquid were centrifuged for 30 min at 3000 rpm and 4 °C, and the pellets were resuspended in 1 mL of lysis buffer according to Sambrook *et al.* (*21*). A volume of 100 μL of a 10 mg/mL proteinase K solution (Sigma, St. Louis, MO) was added to the suspensions, which were then incubated on a linear shaker at 42 °C overnight. An equal volume of phenol was added to 1 mL of the digested suspensions, then mixed for 3 min and centrifuged for 30 min at 14 000 rpm and 4 °C. Supernatant was transferred in a new tube and solvent extraction was repeated twice more using phenol and chloroform in 1:1 volume ratio. DNA was pelleted by adding sodium acetate (3 M, pH=5.2) and ethanol and centrifuging for 30 min at 14 000 rpm and 4 °C. The pellets were finally suspended in 200 μL of double distilled water. DNA quality was controlled by agarose gel electrophoresis and spectrophotometric measurement.

DNA extraction from cheese matrix

Samples of mixes (two weeks after cheese-making) were cut into small pieces with sterile disposable cutters and weighed in order to obtain 5-g aliquots. Aliquots were transferred into sterile 12-mL tubes along with 1-mL of lysis buffer (*21*) and 100 μL of a 10 mg/mL proteinase K solution.

DNA from cheese matrix was obtained by performing phenol/chlorophorm as described above, followed by Nucleospin Food kit purification (Macherey-Nagel, Düren, Germany) in accordance with manufacturer's instructions.

Primers

Primers for PCR amplification of bovine mitochondrial DNA were designed in a region of cytochrome oxidase subunit I (COI gene), which was selected by aligning bovine and buffalo mitochondrial sequences (GenBank accession No. AY488491).

Forward and reverse primers span the positions 134–153 and 246–267, respectively. The following primers were used: BT3 forward, 5′-GAACTCTGCTCGGAGACGAC-3′; BT4 reverse 5′-AGCACCAATTATTAGGGGAAC-3′.

PCR amplification and analysis of results

PCR amplification was performed in a 50 μL reaction volume containing 50 ng DNA, 200 μM dNTPs, 10 mM Tris-HCl, pH=8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 nM primers, 1.5 U DNA polymerase (AmpliTaq Gold, Perkin Elmer). The PCR reaction was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer), using the following conditions: an initial denaturation step at 95 °C for 10 min followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s; final extension step was performed at 72 °C for 10 min. Amplicons were stored at 15 °C before electrophoresis. Positive (DNA from bovine blood) and negative (DNA from water buffalo blood) control samples were run in each amplification.

Results and Discussion

The extraction method used to isolate DNA from Mozzarella cheese matrix and governing liquid showed good DNA yield and quality. Extracted DNA was used as a template to amplify the bovine cytochrome oxidase

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Bos t. Cox1    121 ttaggccaacccggaactctgctcggagacgaccaaatctacaacgtagttgtaaccgca 180
                   || || |||||||||||| |||||||||| |||||||||||||||||||||||| |||
Water b. Cox1  121 ttgggtcaacccggaaccctgctcggagatgaccaaatctacaacgtagttgtaactgca 180

Bos t. Cox1    181 cacgcatttgtaataatcttcttcatagtaataccaatcataattggaggattcggtaac 240
                   ||||||||||||||||||||||| |||||||| ||||| |||||||||||| ||||||||
Water b. Cox1  181 cacgcatttgtaataatcttctttatagtaatgccaattataattggagggttcggtaat 240

Bos t. Cox1    241 tgacttgttcccctaataattggtgctcccgatatagcatttccccgaataaataatata 300
                   ||||| |||   |||||||||| || ||||| |||||||| ||||  ||||||||||||
Water b. Cox1  241 tgactagttaatctaataattggcgcccccgacatagcattcccccagataaataatata 300
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Fig. 1. Sequence comparison between the bovine mitochondrial DNA amplified region (134 bp) and the water buffalo orthologous one (GenBank Accession No. AY488491). Primers' sequences are highlighted

subunit I specific fragment. Species discrimination was based on a new 134 bp amplification fragment (22).

Primers' functionality and specificity were tested with samples of DNA isolated from water buffalo and bovine blood (Fig. 1). In addition, a nucleotide-nucleotide BLAST similarity search (http://www.ncbi.nlm.nih.gov/BLAST) was conducted with the bovine-specific primer sequences to check the absence of unintended matches with buffalo's genome.

A calibration curve was built using cheese matrix samples containing known amounts of bovine milk, ranging from 0.5 up to 30 %. The fluorescence intensity of PCR products is shown in Fig. 2; a direct relation between bovine milk proportion and band intensity was observed, according to the data by Maudet and Taberlet (*15*). Image analysis was performed using an image capture device and dedicated software (Bio-Rad Quantity One®) in order to confirm the linear trend (data not shown).

After these initial assays, the test was applied to the corresponding samples of Mozzarella's governing liquid. Fig. 3 shows the results obtained from the amplification of the DNA extracted from the governing liquids of the mixes six hours after the cheese-making (Fig. 3a),

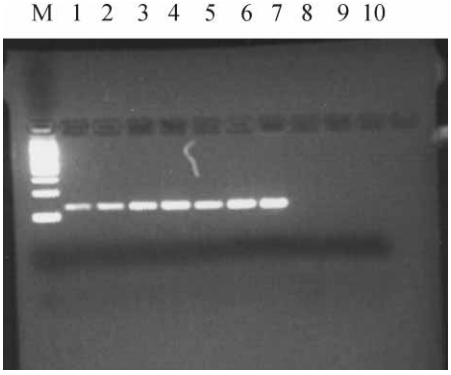

Fig. 2. Separation of PCR products after the amplification of a partial sequence of mtDNA control region (134-bp) extracted from cheese matrix. Amplicons were separated in a 3 % agarose gel containing ethidium bromide. Lane M, 100 bp DNA Ladder (BioLABs, New England); lanes 1 to 6 correspond to the mixtures of water buffalo Mozzarella cheeses containing 0.5, 1, 5, 10, 20 and 30 % of bovine milk, respectively; lane 7, positive control; lane 8, negative control; lane 9, PCR master mix; lane 10, double distilled water

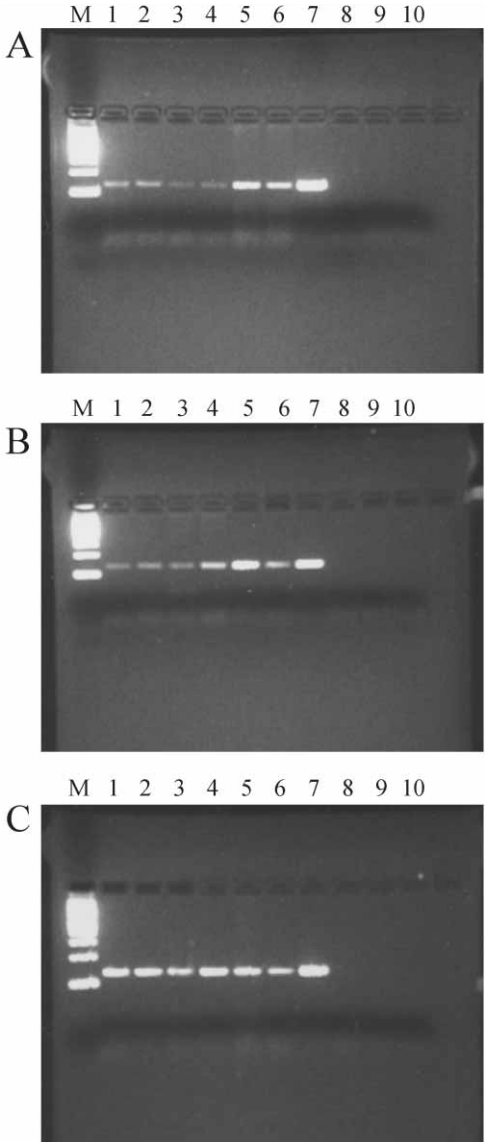

Fig. 3. Polymerase chain reaction of mtDNA from the governing liquids of the mixes analysed six hours (**A**), one week (**B**) and two weeks (**C**) after cheese-making. Lane M, 100 bp DNA Ladder (BioLABs, New England); lanes 1 to 6 correspond to the governing liquids of water buffalo Mozzarella cheese mixtures containing 0.5 to 30 % of bovine milk respectively; lane 7, positive control; lane 8, negative control; lane 9, PCR master mix; lane 10, double distilled water

one week after the cheese-making (Fig. 3b) and two weeks after the cheese-making (Fig. 3c), respectively.

The presence of DNA in governing liquid arises from the cheese matrix's slow flaking that occurs during the whole preservation period. This process seems to take place in a different fashion for each sample, affecting DNA quantity in each governing liquid at all times. Therefore, although the bovine/buffalo ratio is well known for cheese matrix, a direct quantitative relationship between bovine milk percentage and band intensity could not be observed in the governing liquid. This notwithstanding, a clear amplification at the chosen detection limit of 0.5 % was achieved in all three lots examined.

Even if at present bovine milk in water buffalo Mozzarella is tolerated up to 1 % (*23*), due to involuntary cross-contaminations during cheese-making that occur more frequently than deliberate adulteration of the cheese, we chose the detection limit of 0.5 % in order to demonstrate the reliability of the method.

Despite the lack of an evident relation between bovine milk content and band intensity, the latter clearly increases through time (*i.e.* from six hours to two weeks after the cheese-making) for all samples, confirming the link between cheese flaking and DNA quantity in the governing liquid.

DNA from ten commercial PDO water buffalo Mozzarella samples' governing liquids was extracted. Bovine DNA was detected in one sample; the presence of bovine DNA in the positive sample was confirmed by a second extraction from the governing liquid and duplicate amplification; further confirmation was achieved by amplifying DNA extracted from the cheese matrix.

Conclusions

The need to protect both producers and consumers from this fraud prompted the development of several chromatographic (*1*), electrophoretic (*2,3*), immunological (*4*) and mass spectrometry (*5*) analytical techniques, most of which rely on the protein analysis to discriminate the two species. To date, methods based on the isoelectrofocusing of γ-caseins after plasminolysis (*2*) and on HPLC (*1*), which are the official methods in EU and in Italy respectively (*23,24*), have a minimum detection limit of 1 % of bovine milk.

Modern molecular techniques based on DNA analysis have found good applicability in detecting adulteration, and they represent valid complements to the methods relying on protein analysis for the identification of animal species. DNA-based techniques have become effective and reliable also for commercial dairy products (*6,7*).

The PCR assay described here is suitable for routine testing of commercial water buffalo Mozzarella cheese, it is fast, easy and applicable for the detection of DNA from bovine milk, frequently used in adulteration, up to the limit of 0.5 %. This limit was chosen considering the current European law (*23*) tolerating 1 % contamination level. As it is appropriate for testing the cheese matrix, it is also suitable for bovine DNA detection in governing liquid.

Acknowledgments

Authors acknowledge the Caresana dairy for the production of experimental Mozzarellas.

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Detektiranje patvorenja talijanskog sira Mozzarella upotrebom mitohondrijalne DNA kao biomarkera

Sažetak

U mljekarskoj se industriji velika važnost pridaje praćenju patvorenja izvornog talijanskog sira Mozzarella kravljim mlijekom. U ovom je radu cilj bio razviti metodu polimerazne lančane reakcije kako bi se mogla detektirati specifična sekvencija mitohondrijalne DNA goveda u siru Mozzarella. DNA je izolirana iz sira i iz sirutke ekstrakcijom organskim otapalima i zatim pročišćena. Umnožen je fragment dug 134-bp pomoću specifičnih »primera« goveda dizajniranih prema sekvenciji goveđe i bivolje mitohondrijalne citokrom oksidaze, podjedinice 1. Specifičnost »primera« ispitana je korištenjem DNA iz krvi bivola i goveda, jer su njihove DNA prisutne u patvorenim sirevima Mozzarella. Metodom se može pouzdano detektirati udjel od čak 0.5 % kravljeg mlijeka, što ju čini prikladnom i za rutinske analize.