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Detection of Cow Milk in Water Buffalo Cheese by SYBR Green Real-Time PCR: Sensitivity Test on Governing Liquid Samples

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Abstract: A real-time polymerase chain reaction (PCR) assay was developed to detect a bovine-specific mitochondrial DNA sequence in "buffalo" Mozzarella cheese by using primers targeting the cytochrome oxidase subunit 1 (COI) gene. Hot-start PCR, primer design, annealing and signal acquisition temperatures were exploited to obtain reliable analytical conditions which yielded a 134-bp amplicon from cow's DNA only. Water buffalo's DNA didn't originate any amplification product. DNA isolated from blood was used to test primers' specificity and to construct a calibration curve in order to quantify bovine DNA concentration in governing liquid. The method was capable to detect low amounts of cow's DNA in governing liquid samples. Due to low detection limit and fast, simple execution, the analytical protocol described in this work is suitable to become a common tool to detect the fraudulent addition of cow milk in water buffalo Mozzarella cheese.

Key words: Real-time PCR, sensitivity test, species identification, mitochondrial DNA, Mozzarella cheese

Introduction

Italian Mozzarella is a PDO "pasta filata" cheese made using water buffalo milk only and natural whey cultures as fermentation starters. It is produced in Southern Italy since ancient times with little or no changes in the main technological aspects, thus representing a major traditional and genuine Italian dairy product. Cow milk's lower cost and larger availability deriving from greater productions, in conjunction with the frequent breeding of both cows and buffalos in the same farm, encouraged the undeclared addition of cow milk in violation of the Italian law and the European PDO certification (Anonymous, 1996).

The need to protect both producers and consumers from this fraud prompted the development of several chromatographic (Pellegrino *et al.*, 1991), electrophoretic (Addeo *et al.*, 1989; Cartoni *et al.*, 1998), immunological (Addeo *et al.*, 1995) and mass-spectrometric (Cozzolino *et al.*, 2002) analytical techniques, most of which rely on protein analysis to discriminate the two species. To date, methods based on the isoelectrofocusing of gamma-caseins after plasminolysis (Addeo *et al.*, 1989) and based on HPLC (Pellegrino *et al.*, 1991), which are the official methods in EU (Anonymous, 2001) and in Italy respectively (Gazzetta Ufficiale della Repubblica Italiana, 1996), have a minimum detection limit of 1 % cow milk.

In the last years, some molecular methods for bovine species identification, mostly based upon PCR technology (Bardin *et al.*, 1994; Lopez-Calleja *et al.*, 2004) were presented. The DNA assays consist essentially in extracting DNA from governing liquid (Lipkin *et al.*, 1993) and amplifying a mitochondrial *cyt b* locus (Kocher *et al.*, 1989), while species identification

relies upon PCR reaction multiplexing by means of a primer pair for each one (Branciari *et al.*, 2000; Rea *et al.*, 2001; Bottero *et al.*, 2002).

We previously reported bovine species discrimination in water buffalo Mozzarella by PCR (Feligini *et al.*, 2005).

We now describe a real-time PCR assay capable to detect bovine DNA in water buffalo Mozzarella cheese. The most important aim of this work was to demonstrate the high potential of this technique in frauds' recognition. Real-time PCR uses a fluorescence detection system that can collect fluorescence measurements during the amplification cycles, thus monitoring the specific product's accumulation, and, unlike conventional PCR, relate them to template's initial quantity. This SYBR green I-based assay allows the simple and fast detection of a specific 134-bp amplicon located in the bovine mitochondrial cytochrome oxidase subunit 1 (COI) gene in samples containing down to 0.5 % of cow milk.

Materials and Methods

Samples: Experimental water buffalo Mozzarella cheeses were manufactured in a traditional fashion, except for adding bovine milk in 30, 20, 10, 5, 1 and 0.5% proportions. The governing liquid (i.e. the pickle in which Mozzarella is packaged) of experimental samples was preserved at -20°C.

Water buffalo and bovine reference DNA were extracted from blood using phenol and chloroform (1:1 vol/vol) (Sambrook *et al.*, 1989). DNA was precipitated by adding 3 M sodium acetate at pH = 5.2 and absolute ethanol (1:2.5 vol/vol), then washed with 100 µL of an ethanol/double distilled water solution (70:30 vol/vol) and finally suspended in distilled water. DNA from blood

was spectrophotometrically quantified in a GeneQuantPro apparatus (Amersham Pharmacia Biotech, Uppsala, Sweden) at 260/280 nm. After DNA extraction, samples were preserved frozen at -20°C.

DNA extraction: Governing liquid (40 mL) was centrifuged (4°C) for 30 min at 2000 x g and the pellets were resuspended in 1 mL lysis buffer (1 mM EDTA, 10 mM Tris/HCl pH 7.5, 50 mM NaCl, 5 g/L SDS). 100 µL of a 10 mg/mL Proteinase K (Sigma-Aldrich, St. Louis, MO) were added to the solution, which was then incubated on a linear shaker at 42°C overnight. 1 mL of the digested suspensions was solvent-extracted once using 1 mL phenol and three times using a phenol/chloroform (1:1 vol/vol) volume equal to the supernatant's one, each time followed by centrifugation for 15 min at 18,000 x g and transfer of the supernatant to a new tube. DNA was pelleted by adding sodium acetate (3 M, pH 5.2) and absolute ethanol (-20°C), incubating for 120 min at -20°C and centrifuging (4°C) for 30 min at 18,000 x g. The pellet was finally resuspended in 200 µL sterile deionized water and frozen until analysis.

Real-time PCR amplification: The real-time PCR amplifications were performed in a DNA Engine Opticon® 2 System for continuous fluorescence detection (MJ Research, Boston, USA). The reaction was conducted in a final volume of 30 µL containing: 200 µM dNTPs, 10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 100 nM primers, 0.9 U DNA polymerase (AmpliTaq Gold, Perkin Elmer) and 6 µL of DNA solution an unknown concentration. The primer pair consisting of primer BT3 [5'-GAACTCTGCTCGGAGACGAC-3'] and primer BT4 [5'-AGCACCAATTATTAGGGGAAC-3'] was used for the amplification of a bovine-specific 134-bp region of the COI gene (Feligini *et al.*, 2005). Thermocycling was performed as 45 cycles of 30 s at 95°C (denaturation), 30 s at 56°C (annealing) and 30 s at 72°C (extension). The final extension step was carried out as 5 min at 72°C. SYBR Green I (Stratagene, La Jolla, CA, USA) was used to monitor the accumulation of double-stranded 134 bp amplicon; it was added to the PCR reagents as a 1:2,000 aqueous solution in order to obtain a final concentration of 1:50,000. The dye was diluted prior to each run because of its instability to freezing and thawing according to Wittwer *et al.* (2001). PCR negative controls were run along with samples, in order to detect false positives due to possible contaminations.

Results of the real-time PCR assay were analyzed with Opticon® 2 software supplied with the Opticon cycler, obtaining fluorescence intensity vs cycle number curves.

Calibration curve: After spectrophotometrical quantification, reference bovine DNA extracted from blood was diluted to obtain a series of concentration

standards set at 50 ng/µL, 5 ng/µL and 0.5 ng/µL respectively. Standards were amplified four times along with samples, in triplicate for each run. Fluorescence data from all standards' amplifications were processed in order to construct a concentration vs Ct semilog calibration curve. This calibration curve was then used to quantify samples from governing liquid.

Results and Discussion

A real-time polymerase chain reaction amplifying a fragment of the cytochrome oxidase subunit I (COI) gene was developed for the detection of bovine milk in buffalo Mozzarella cheese. Primers' specificity and functionality were tested by amplifying reference DNA from blood of both cow and water buffalo. The amplification plot, showing the changing in measured fluorescence along PCR cycles, is reported in Fig. 1. The former sample showed a clear increase in emitted fluorescence starting at cycle 24.16, while the latter one didn't reach the threshold intensity. PCR products were subsequently analyzed by agarose gel electrophoresis and sequencing in order to obtain further confirmation regarding the expected size (134 bp) of the specific amplicon and the absence of nonspecific amplification products.

Fig. 2a shows the fluorescence profiles obtained from one of the four standards' triplicate amplifications. The observed average threshold cycle (Ct) values were 22.84, 26.89 and 30.33 and were inversely related with standards' concentration.

Mean Ct and confidence interval (P = 0.05) were calculated for each concentration from all standards runs' data (Table 1). Standards' fluorescence profiles and Ct values were evidently grouped according to their concentration (Fig. 2a) and the calibration curve featured a good linear correlation coefficient (Fig. 2b), notwithstanding the confidence intervals slightly widened along with concentration's lowering.

Logarithms of the DNA concentration were plot against the calculated means (Fig. 2b), obtaining a straight line of equation $y = -0.27087x + 8.1669$ (where y is the log of DNA concentration and x is the Ct), with a linear correlation coefficient (r^2) of 0.99993. This equation was used to quantify DNA from governing liquid.

To evaluate method's effectiveness and reliability on DNA isolated from governing liquid, duplicate real-time PCR amplifications were performed. Fig. 3 shows the fluorescence profiles obtained in a single run. Although the bovine-to-buffalo ratio was known, the DNA concentration in samples was not assessed prior to PCR reaction whilst it was calculated by means of the Opticon Monitor® software applying the calibration curve. The estimated concentrations of bovine DNA from governing liquid samples were low (down to the calibration curve's limit of 0.5 ng/µL) and very close to each other regardless to the bovine-to-buffalo ratio, thus

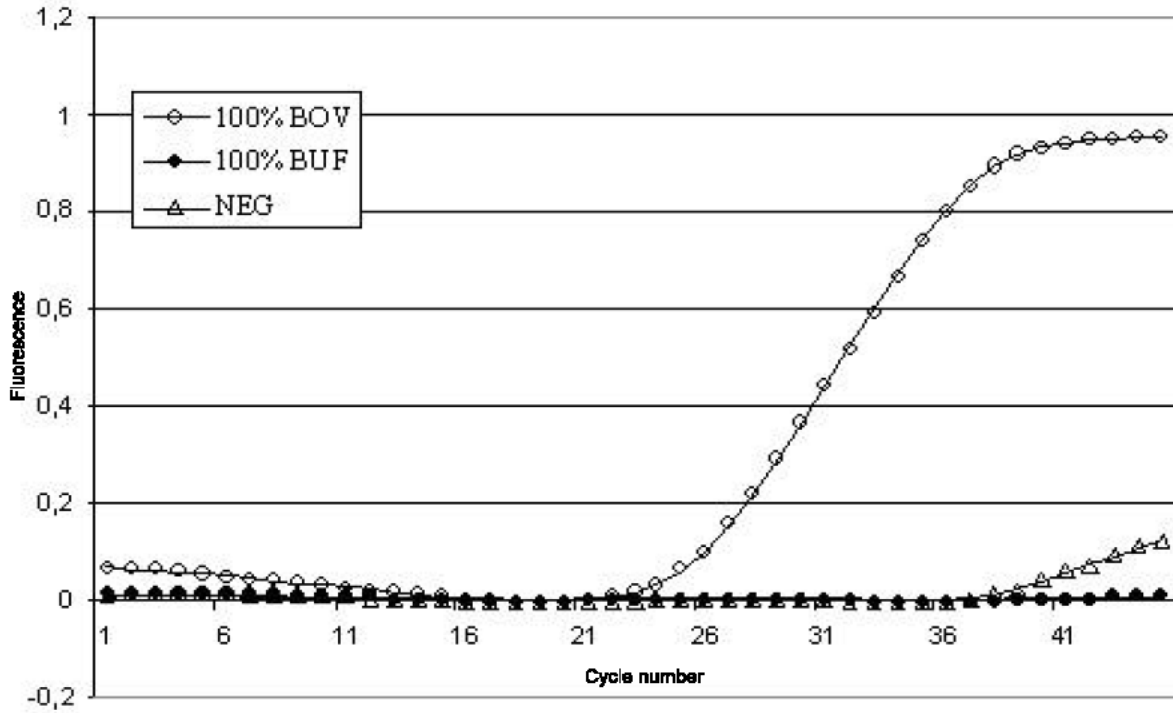


Fig. 1: Real-time PCR of bovine and water buffalo reference DNA isolated from blood. The reaction was carried out using BT3/BT4 bovine-specific primer pair. SYBR Green I fluorescent dye was used to monitor the amplification of a 134-bp fragment in CO1 gene from mitochondrial bovine DNA.

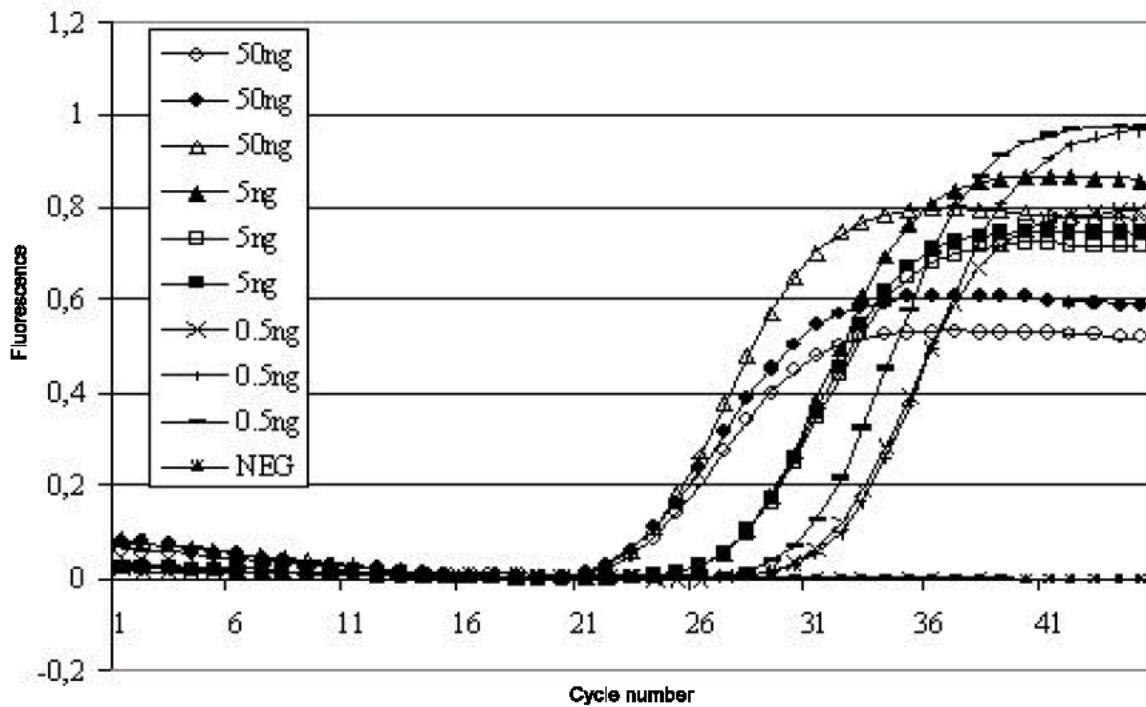


Fig. 2 a: Fluorescent profiles obtained from one triplicate real-time PCR of bovine DNA standards (50, 5 and 0.5 ng/ μ L).

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Table 1: Relation between bovine DNA standards' concentration and threshold cycle (Ct)

Concentration (ng/μl)	Repeats	Ct (P = 0.05)
50	12	23.90 ± 0.48
5.0	12	27.54 ± 0.62
0.50	11*	31.28 ± 1.08

*Plus one false negative

Table 2: Quantification of DNA samples from governing liquid by SYBR Green-based real-time PCR

Sample	Ct (P = 0.05)	Bovine DNA ¹ (ng/μl)	Total DNA ² (ng/μl)
0.5% bovine	31.26 ± 0.19	0.5	100.15
1% bovine	28.29 ± 0.70	3.19	319
5% bovine	28.8 ± 1.1	2.36	47.1
10% bovine	29.2 ± 1.1	1.8	18
20% bovine	29.8 ± 1.5	1.25	6.3
30% bovine	25.26 ± 0.25	21.2	70.5

¹Calculated from mean Ct by applying the calibration curve

²Calculated dividing bovine DNA content by bovine proportion reported in "Sample" column

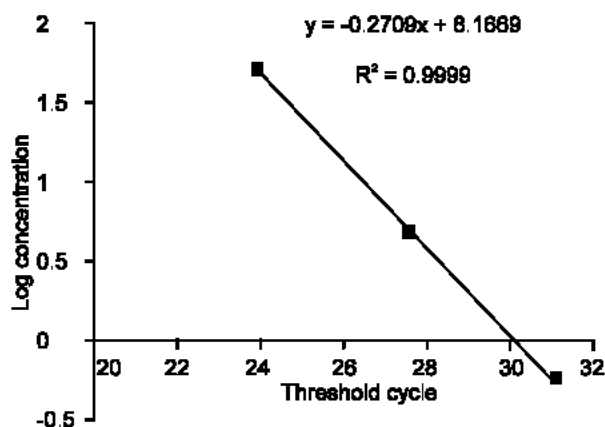


Fig. 2b: Standard curve obtained by plotting the logarithm of the DNA concentration vs threshold cycle (Ct) mean values. The curve was constructed using data from all the four triplicate standards' amplifications.

resulting in a wide range of calculated extraction yields (Table 2). The small DNA concentration loaded confirmed the real-time PCR's high sensitivity and low detection limit.

The analytical strategy we describe in this work differs from the ones developed by other authors focusing on bovine DNA detection in milk (Maudet and Taberlet, 2001; Lopez-Calleja *et al.*, 2004) or other matrices (Tartaglia *et al.*, 1998; Herman, 2001; Lahiff *et al.*, 2002) for primers' target sequences and for the real-time PCR experimental approach. We chose to analyze DNA samples from governing liquid at unknown concentration, performing "inline" absolute quantification by means of the calibration curve instead of

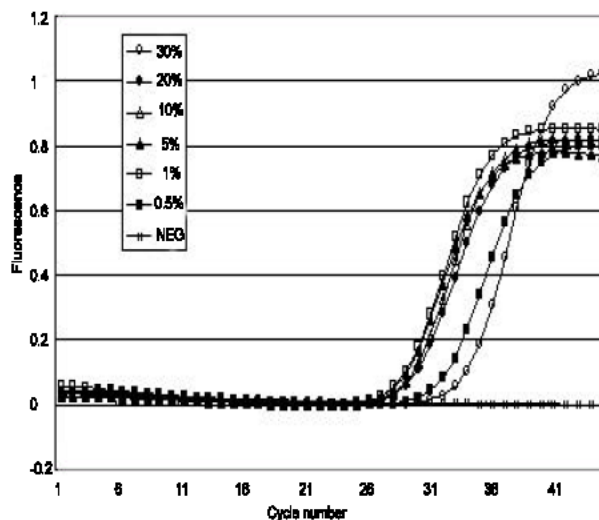


Fig. 3: Fluorescence profiles obtained by real-time PCR of DNA samples isolated from governing liquid of experimental cheeses manufactured using different percentages of cow milk.

spectrophotometrical one prior to analysis, in order to speed up the method and to verify its reliability in routine applications. This implies that samples were not normalized for total DNA concentration before the amplification. Because of this, a direct relation between the percentage of added bovine milk and the absolute DNA concentration was not expected (Fig. 3). Samples' fluorescence profiles and Ct values resulted to be markedly similar, implying that loaded amounts of bovine DNA were very close to each other in spite of the corresponding bovine-to-buffalo ratios. Since DNA quality and yield are among the most important variables in real-time PCR applications, obtaining DNA susceptible to be amplified from governing liquid represents this work's first result. The presence of DNA in governing liquid is due to the cheese matrix's exfoliation that slowly occurs during the whole preservation period. DNA was found in all experimental samples. Real time amplification of DNA from governing liquid proved the method's actual applicability for species detection purposes. Hot-start PCR and fluorescence signal acquisition were optimal at 56°C, allowing SYBR Green I-based real-time PCR to be sensitive and specific (Morrison *et al.*, 1998), although amplification was obtained in conditions of greater stringency as well.

The possibility to detect small quantities is important, in addition to recognizing frauds, also in protecting consumers allergic to cow milk proteins. Due to its low detection limit, the assay here described is suitable to routine analysis and can be applied to detect fraudulent cow milk addition in cheeses. In the future, this real-time PCR application could be extended to the identification of frauds in other kinds of cheese.

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