JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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

ROSA M. LOPPARELLI, BARBARA CARDAZZO,* STEFANIA BALZAN, VALERIO GIACCONE, AND ENRICO NOVELLI

Dipartimento di Sanità Pubblica, Patologia Comparata e Igiene Veterinaria, Università di Padova, AGRIPOLIS 35020 Legnaro, Italy

Mozzarella cheese obtained from buffalo (*Bubalus bubalis*) milk is a typical Italian product certificated by means of the European Protected Designation of Origin (PDO). Mozzarella cheese can also be obtained from bovine milk or bovine/buffalo milk mixtures, but in this case, it cannot be sold as PDO product, and its label must report the actual ingredients. However, bovine milk in PDO products was frequently detected in the past, suggesting fraudulent addition or accidental contamination. Several methods based on end-point polymerase chain reaction (PCR) have been profitably applied in a large number of tests to detect the presence of undeclared ingredients, also in dairy products. In the present study we report a real-time PCR method able to quantify bovine milk addition to pure buffalo cheese products. We validated a normalized procedure based on two targets: bovine mitochondrial cytochrome *b* (cyt *b*) to detect and quantify the bovine DNA and nuclear growth hormone (GH) gene used as a universal reference marker. With the use of this real-time PCR assay, 64 commercial mozzarella di bufala cheese samples purchased at local supermarkets, dairy shops, or directly from cheese manufacturers were analyzed. The results obtained demonstrate that most of the commercial samples were contaminated with bovine milk. Therefore, this assay could be conveniently employed to carry out routine and accurate controls aimed not only to discourage any fraudulent behavior but also to reduce risks for consumer health.

KEYWORDS: Dairy products; bovine milk; real-time PCR; species identification; cyt *b*

INTRODUCTION

Mozzarella cheese obtained from buffalo (*Bubalus bubalis*) milk is a typical Italian product marketed all over the world certificated by the European Protected Designation of Origin (PDO) (*1*), whose first production marks in south Italy probably go back to the 12th to 13th century.

Nowadays, total production is continuously growing (nearly 28 tons in 2004), as well as exportation (17.5% in the same year). This growth has been supported, in the last 50 years, by the increasing number of reared buffaloes. Production is mainly concentrated in three Italian regions: Campania (87.9%), Latium, and Apulia.

Since 1981 a voluntary association of producers (164 in 2004) stated specific rules about cheese making according to traditional practices: only fresh, raw or pasteurized, buffalo milk can be used, acidification should be carried out by means of natural (not commercially selected) whey cultures, while bovine rennet is allowed.

Anyway, mozzarella cheese can also be obtained from bovine milk or bovine/buffalo milk mixtures, but in this case, it cannot be sold as the more expensive PDO product, and its label must report the actual ingredients. On the other hand, bovine milk in PDO product was detected in the past suggesting a fraudulent addition or an accidental presence due, for instance, to contamination in the farm or later in the cheese factory if both species are treated.

Animal species identification in dairy products, such as buffalo mozzarella, has become more and more important, not only with regards to accurate consumer information and legal aspects (concerned with labeling and guarantee requirements) but also to public health (cow caseins are known allergens even if present in very low quantity).

The official control method (*2*) to detect bovine proteins in dairy products is based on isoelectrofocusing (IEF) of γ-caseins after plasminolysis.

Even if this technique is declared reliable for thermally treated cow milk, some authors observed that it can carry out uncertain results in severe heat-treated cheese (*3*) and in the case of weak contamination.

* Corresponding author. Phone: 39-049-8272965; 39-049-8272604. E-mail: barbara.cardazzo@unipd.it.

10.1021/jf0637271 CCC: $37.00
Published on Web 04/10/2007

Other analytical techniques for animal species identification have been developed: ^{13}C NMR (nuclear magnetic resonance) on triacylglycerols to distinguish cow and buffalo in milk (*4*), an HPLC technique for β-lactoglobulins (β-lg) analysis on buffalo milk and mozzarella cheese (*5*), capillary electrophoresis and acrylamide gels (*6*, *7*) coupled with a multivariate regression analysis (*8*), and more recently, immunological methods for detection of species adulteration based on enzyme-linked immunoadsorbent assay (ELISA) (*9*). However, the main restrictions of these methods are the lack of suitability for routinely utilization, the long time needed, and the lack of absolute specificity (*10*, *11*).

Methods based on polymerase chain reaction (PCR) (*10*, *11*) have been profitably applied in a large number of tests for animal species identification, also in dairy products: RFLP analysis of PCR products, duplex-PCR, multiplex-PCR, terminal-RFLP based on PCR and capillary electrophoresis, and SNuPE (single nucleotide primer extension) based on the detection of species-specific single-nucleotide polymorphism (SNPs) of a marker gene (*3*, *12*, *13*). These methods are based on the persistence of genomic DNA extracted from somatic cells both in milk and cheese, even if highly ripened (*3*, *12*, *14*, *15*).

The most common assays for species identification are based on PCR analysis of species-specific mitochondrial DNA (mtDNA) sequences. In fact, mtDNA can be considered as a naturally amplified source of genetic variability (*16*). Other techniques are based on nuclear DNA sequences, like 28S and 18S rRNA multicopy genes (*12*) or β-caseins genes (*17*), well-known markers for animal identification of dairy products. End-point PCR assays for bovine milk contamination in buffalo cheese have been previously reported (*3*, *18*, *19*). Mafra et al. (*20*) applied a semiquantitative approach based on a duplex end-point PCR of 12S and 16S rRNA mitochondrial genes for quantification of raw, pasteurized, and powered bovine milk in different types of ovine cheese. However, a quantitative real-time PCR approach might be more suitable to distinguish between fraudulent addition of bovine milk and cross-contamination or simply bovine rennet employment. Real-time PCR is widely used to quantify GMO in various types of crops and foodstuff (*21*, *22*) and to evaluate the presence of DNA of various animal species in meat preparations (*16*, *23*, *24*) or in typical products (*25*).

In the present study we report a real-time PCR method able to quantify bovine milk addition to pure buffalo cheese products, such as PDO buffalo mozzarella, using a normalized procedure based on two targets: mitochondrial cytochrome *b* (cyt *b*) and nuclear growth hormone (GH) genes; the second was selected as a single-copy and conserved gene between the different species of interest, cow and buffalo.

MATERIALS AND METHODS

Sample Collection and Standard Preparation. Blood, milk, and cheese (standard and commercial samples) were tested. Blood samples from cow (*Bos taurus*), two specimens each for Brown Swiss, Burlina, Rendena, and Holstein breeds and water buffalo (*B. bubalis*) were collected in EDTA-containing vials and stored at −20 °C.

Samples of bulk cow (Holstein breed) milk were collected in a farm located in the Venetian region of Italy. Four separate samplings were carried out in summer and six in autumn 2005. Fifty grams of each milk sample were centrifuged at 4000 rpm, and the cell pellet was stored at −20 °C for later use.

Standard samples, i.e., 100% bovine milk mozzarella cheese and 100% buffalo milk mozzarella cheese, were produced accordingly to the procedure of PDO Mozzarella di Bufala Campana regulation (*26*), with minor adaptations to the laboratory equipment. Milk used in the production of standard cheeses was sampled in summer 2005, as previously described. The standard samples were lyophilized and accurately weighted to obtain "mixed" samples with 0.1%, 0.6%, 1%, 2%, 5%, 10%, and 20% (w/w), respectively, of cow matter in buffalo cheese.

These standard samples were weighted and prepared twice. Aliquots of 200 mg of each mix standard sample were frozen at −20 °C for later use. Two other standard cheeses made of 100% buffalo milk were produced using lyophilized commercial whey starters.

Commercial mozzarella cheese samples were purchased at local supermarkets and dairy shops or directly from cheese manufacturers. Two aliquots of 200 mg of each sample were frozen at −20 °C. In total, 2 samples of commercial bovine mozzarella cheese and 64 samples of commercial buffalo mozzarella cheese (48 PDO and 16 not PDO for a total of 37 different brands) were collected, 28 during the summer and 36 in the autumn 2005. (Most of the commercial labels were purchased both in summer and in autumn.)

DNA Extraction. DNA was extracted from 200 μL of blood samples using Invisorb Blood Mini Kit (Invitek, Berlin, Germany), from milk cell pellets after centrifugation using Invisorb Cell Mini Kit (Invitek), and from 200 mg of mozzarella cheese using Invisorb Tissue Mini Kit (Invitek). All extractions were carried out following the instructions provided by kit manufacturer.

Design of Reference and Species-Specific Primers and Probes. The nucleotide sequence of mRNA coding for GH of *B. taurus* and *B. bubalis* were aligned using the ClustalX program (*27*). Exon−exon junctions were located on bovine coding sequence based on the information contained in the Genome Browser database (Btau_2.0, http://genome.ucsc.edu). A conserved region of the fourth exon sequence was used to design universal primers and TaqMan minor groove binding (MGB) probe with the Primer Express 2.0.0 software (Applied Biosystems, Foster City, CA). The sequences of primers and probe were as follows: GH_F, 5′-TTGGGCCCCTGCAGTTC-3′, GH_R, 5′-GGTCCGAGGTGCCAAACAC-3′, GH_MGB 5′-AGCAGAGTCTTCACCAAC-3′. Species-specific primers (Cyt*b*BOS) and MGB probe for the cyt *b* gene of *B. taurus* were those previously reported (*23*).

Real-Time PCR Amplification. Real-time PCR amplification was performed on an ABI PRISM 7000 thermocycler (Applied Biosystems), using default settings, in a final volume of 20 μL, including 0.5, 1, or 2.5 μL of DNA template extracted from blood, milk, and mozzarella samples, respectively. The SYBRGreen I Master Mix 1X (Applied Biosystems) was used together with 250 nM of each primer. The TaqMan Universal PCR Master Mix 1X (Applied Biosystems) was used with 500 nM of each primer and 200 nM of TaqMan MGB probe. Outputs of real-time amplifications were analyzed by means of SDS 7000 1.1 software (Applied Biosystems). The fluorescence signal in each reaction was plotted against cycle number, and the threshold cycle (Ct) was calculated using the default threshold line. All PCR reactions aimed to evaluate the specificity, efficiency, and sensitivity of the real-time assays were performed in triplicate, whereas reactions done to quantify commercial samples were performed in duplicate. To evaluate the efficiency and the dynamic range of each primer pair, serial 1:5 dilutions were prepared for each DNA template extracted from blood, whereas 1:2 serial dilutions were prepared from each milk and mozzarella cheese sample. Standard curves for absolute quantification were obtained plotting the log percentage of cow contained in each standard sample against the difference between Ct's of the species-specific and the reference reaction ($\Delta Ct = Ct_{CytbBOS} - Ct_{GH}$). Each amplification plate aimed to perform absolute quantification included both test and standard samples.

Statistical Analysis. The evaluation of the differences between relative quantification series of data was performed by a Wilcoxon two-sample test available at www.fon.hum.uva.nl/Service/Statistics/Wilcoxon_Test.html.

RESULTS

Validation of Real-Time PCR Assays. The functionality of both primer pairs (GH and Cyt*b*BOS) and the specificity of the target gene primers were assessed by real-time PCR amplifica-

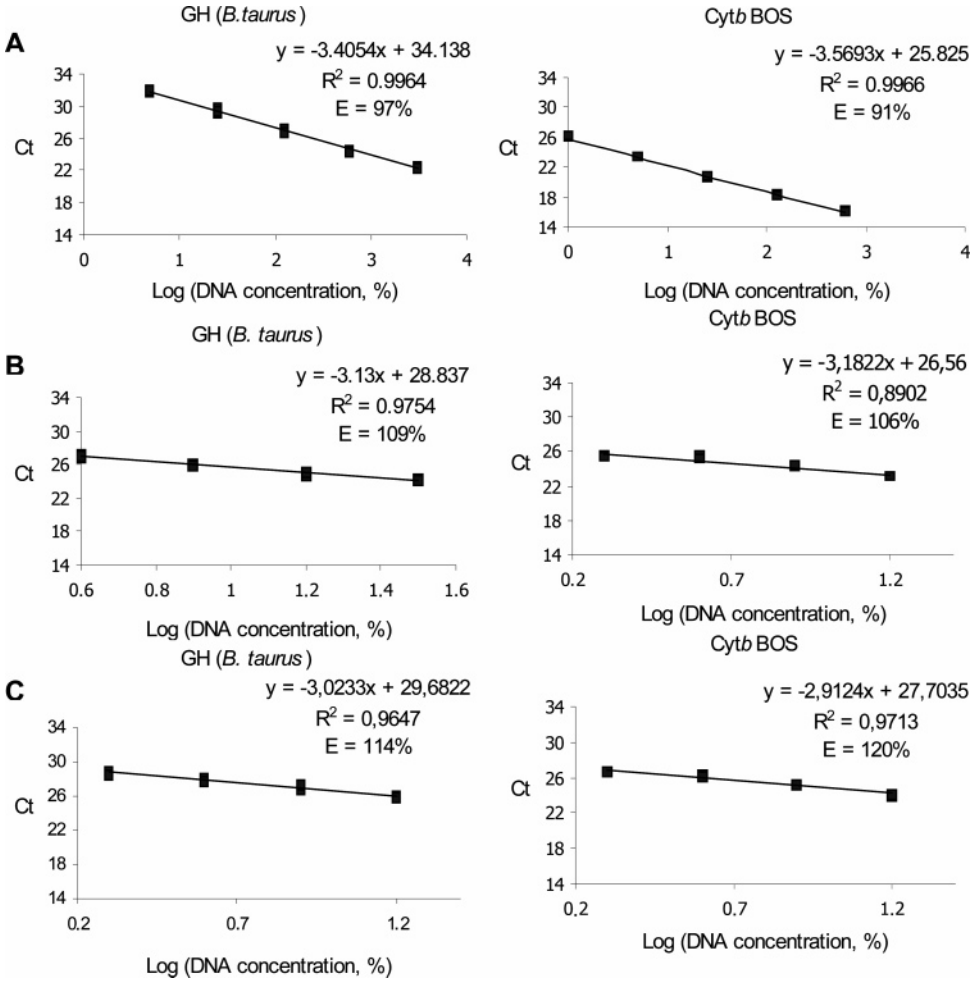

Figure 1. Linearity test and amplification efficiency of the universal reference GH and the species-specific Cyt*b*BOS assays with fluorescent SYBR green I dye on blood (**A**), milk (**B**), and cheese (**C**) templates serially diluted.

Table 1. Slope and Efficiency of the Regression Curve[a,b]

	Assay							
	GH				Cyt*b*BOS			
	SYBR green I			probe	SYBR green I			probe
	blood	milk	cheese	cheese	blood	milk	cheese	cheese
dynamic range (ΔCt)[c]	9.33	2.81	2.72	8.37	9.87	2.36	2.59	12.51
Slope	–3.40	–3.13	–3.02	–3.52	–3.57	–3.18	–2.91	–3.52
efficiency (%)	97.0	109.0	114.0	92.8	91.0	106.0	120.0	92.8

[a] Comparison among different matrixes (blood, milk, and cheese) on GH and Cyt*b*BOS genes using the nonspecific dye SYBR green I and the specific TaqMan MGB probe. [b] The decimal efficiency values are obtained from the slope value: $E = [10^{(-1/\text{slope})}] - 1$. [c] $\Delta Ct = (Ct_{MAX} - Ct_{MIN})$ is referred to serially diluted templates.

tions using SYBR green I as a fluorescent dye on DNAs extracted from blood samples of four different cattle breeds and from water buffalo. No cross-amplification for the Cyt*b*BOS primers was observed. The efficiency of the different assays was evaluated by SYBR green I using the DNA templates extracted from blood, milk, and mozzarella cheese samples. Efficiency and specificity of the GH and Cyt*b*BOS TaqMan MGB probes were evaluated using DNA templates extracted from mozzarella cheese.

The results of the tests with SYBR green I are reported in **Figure 1**. Similar efficiencies were obtained for the reference and the target gene's primer pairs in the same matrix; the efficiencies varied in a range of acceptability. DNA extracted from each cattle breed sample gave comparable results (data not shown).

In **Table 1**, the efficiencies and the dynamic range values of the real-time PCR amplifications with SYBR green I (three matrixes) and with the specific probes (cheese) for both genes are summarized. The probe assay efficiency was 92.8% for both tests with larger dynamic ranges than those obtained with SYBR green I. The specificity of Cyt*b*BOS TaqMan MGB probe was tested amplifying buffalo DNA templates extracted from a 100% buffalo milk mozzarella cheese (standard sample). As for the SYBR green I assay, no amplification was observed. These results indicate that the TaqMan assay on mozzarella DNA samples could be used in quantification analysis.

Evaluation of Mitochondrial Copy Number. A potential drawback of using mitochondrial genes as molecular markers for quantification of species-specific DNAs could be the variation in the number of mtDNA copies per cell across

Table 2. Relative Quantification by the ΔΔCt Value Method of Templates Obtained from Bovine Milk (bm) Samples Collected in Summer ($n = 4$) and in Autumn ($n = 6$)

sample	ΔCt	ΔCt SD	ΔΔCt	RQ	RQ SD
		Summer			
bm 1	–5.86	0.21	–1.24	2.36	1.11
bm 2	–5.67	0.11	–1.05	2.08	0.51
bm 3	–4.62	0.09	0.00	1.00	0.15
bm 4	–4.96	0.08	–0.34	1.27	0.21
summer mean	–5.28	0.58	–0.66	1.68	0.64
		Autumn			
bm 5	–4.97	0.12	–0.35	1.28	0.31
bm 6	–5.36	0.13	–0.74	1.67	0.43
bm 7	–5.29	0.16	–0.67	1.59	0.59
bm 8	–5.76	0.14	–1.14	2.20	0.58
bm 9	–4.67	0.13	–0.05	1.03	0.26
bm 10	–4.12	0.02	0.50	0.71	0.03
autumn mean	–5.03	0.58	–0.41	1.41	0.53
general mean	–5.13	0.18	–0.65	1.52	0.18

samples (bulk milk) collected in different seasons (summer and autumn). An estimate of such variation might be obtained comparing results from the GH assay, which is based on a single-copy nuclear locus, and from the Cyt*b*BOS test, which is based on a mitochondrial gene. ΔCt values ($Ct_{Cyt\,bBOS} - Ct_{GH}$) and the relative quantification (RQ) value between summer and autumn milk samples were not significantly different by using a Wilcoxon two-sample test, suggesting that no relevant variation in mtDNA copy number was present (**Table 2**).

Absolute Quantification Curves and Evaluation of Commercial Samples. All commercial samples as well as buffalo mozzarella samples experimentally produced with addition of commercial whey starters were analyzed using the GH and Cyt*b*BOS TaqMan assay.

The efficiency (96%) was inferred from the slope of the standard curve (data not shown) using separately two different standard sets: 0.6%, 1%, 5% and 5%, 10%, 20% (w/w). The 0.6% value represented the lowest limit for absolute quantification, because below this value the curve was not linear. The lowest detection limit was 0.1% of cow in buffalo mozzarella; therefore, samples showing values lower than 0.1% have been considered negative (N). Values in the range of 0.1−0.6%, indicative of the presence of cow matter, were considered positive, but the amount was not quantifiable (positive not quantifiable, PNQ). Samples with values higher than 0.6% were considered positive (P).

Standard samples of pure buffalo mozzarella made with dehydrated whey were attributed as PNQ. Absolute percentage values of commercial samples are reported in **Table 3**. Percentage data were obtained from 0.6%, 1%, 5% standard curve for samples with results lower than 5% and from 5%, 10%, 20% standard curve for samples higher than 5%; samples with results out of range (>20% and <0.6%) were not quantified. For each sample the value was calculated as the average of the quantification of two independent extraction aliquots. The summary of data (**Figure 2**) indicates that bovine milk was present, in variable amounts, in the majority (P + PNQ = 51/64, 79.7%) of the commercial samples. Of these, 39 were PDO products (76.5%) while 12 were not PDO. Positive samples, where cow milk contamination has been unequivocally quantified, were 37.5% (79.2% of them were labeled PDO). Only 2 of over 37 total different brands were N, considering both sampling seasons.

Table 3. Absolute Quantification of Bovine DNA Content by Means of the ΔCt Method of 64 Water Buffalo Mozzarella Samples[a]

samples		bovine DNA (%)					
		summer			autumn		
brand	PDO	sample	DNA (%) or evaluation	SD	sample	DNA (%) or evaluation	SD
1	yes	1s	3.21	0.01			
2	no	2s	PNQ		2a	PNQ	
3	yes	3s	0.86	0.40	3a	PNQ	
4	yes	4s	1.35	0.39	4a	0.69	0.24
5	yes	5s	N		5a	N	
6	yes	6s	0.66	0.22	6a	PNQ	
7	yes	7s	PNQ		7a	PNQ	
8	no	8s	0.64	0.33	8a	PNQ	
9	yes	9s	PNQ		9a	N	
10	yes	10s	1.10	0.34	10a	PNQ	
11	yes	11s	1.66	0.37	11a	1.88	0.00
12	yes	12s	PNQ		12a	1.34	0.22
13	yes	13s	N		13a	PNQ	
14	yes	14s	PNQ		14a	1.86	0.45
15	no	15s	>20%		15a	PNQ	
16	no	16s	PNQ		16a	0.81	0.24
17	yes	17s	>20%		17a	PNQ	
18	yes	18s	0.75	0.09	18a	PNQ	
19	no	19s	>20%		19a	>20%	
20	yes	20s	0.88	0.05			
21	yes	21s	1.98	0.26	21a	PNQ	
22	yes	22s	N				
23	yes	23s	0.72	0.00	23a	PNQ	
24	yes	24s	3.04	0.39			
25	no	25s	N		25a	N	
26	yes	26s	PNQ		26a	PNQ	
27	no	27s	PNQ		27a	PNQ	
28	yes	28s	1.46	0.82	28a	2.72	1.14
29	yes				29a	PNQ	
30	yes				30a	N	
31	yes				31a	N	
					$31a_1$[b]	0.80	0.18
32	no				32a	N	
					$32a_1$[b]	N	
33	yes				33a	N	
					$33a_1$[b]	PNQ	
34	yes				34a	N	
35	yes				35a	PNQ	
36	yes				36a	PNQ	
37	yes				37a	PNQ	

[a] Results are reported as bovine DNA content (%, w/w) when the sample was positive (P) or as an evaluation when it was negative (N) and positive but not quantifiable (PNQ). See also the text. [b] 31a and $31a_1$ are different lots of production purchased in the same season referred to the same mozzarella brand; the same with sample nos. 32 and 33.

DISCUSSION

The broad diffusion in the world of the typical Italian product named water buffalo mozzarella, already registered since 1996 (*1*) as a PDO product, has economic, qualitative, and legal implications. The most simple fraud is the addition of bovine milk. Nonetheless relevant could be the sanitary implication that the consumption of vaccine caseins can get in people carrying an allergy against them. In the present study, a quantitative real-time PCR method has been carried out in order to quantify bovine DNA in water buffalo mozzarella. Mitochondrial DNA genes are the most widely used markers for animal species identification in foodstuff, due to the presence of multiple copies (10−1000) of mtDNA in each cell. Starting with reduced amounts of DNA (frequent if working with processed food), these targets significantly increase the sensitivity of the assay. However, large variability in the copies number of the mitochondrial genome has been reported among different species, individuals of the same species, or among different tissues of

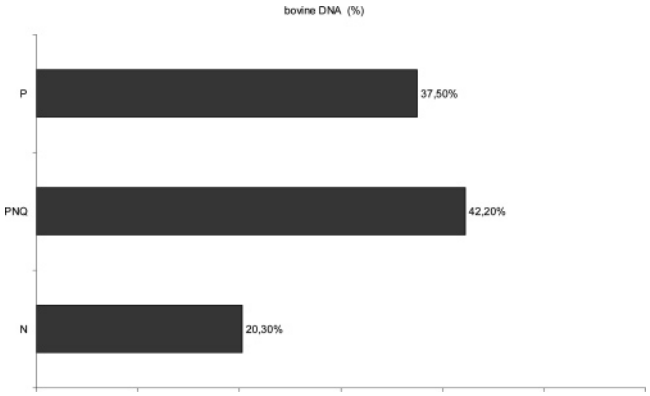

Figure 2. Sharing of positive (P), positive but not quantifiable (PNQ), and negative (N) commercial samples of water buffalo mozzarella ($n = 64$). Results of absolute quantification as a percentage are shown.

the same individual, and not the least, as a consequence of aging (*23*, *28*, *29*). Variation in mtDNA copy number might seriously affect the reliability of real-time PCR quantification of species-specific DNA, especially when an mtDNA marker is used for normalization.

In order to reduce the risk of erroneous quantification due to variation in mtDNA copy number, while maintaining sufficient sensitivity, the use of alternative multicopy nuclear markers was explored. The nuclear ribosomal gene 18S has been proposed in consideration of the limited variability between species (*23*, *30*). Alternatively, the use of repetitive elements has been suggested (*16*, *31*). At present, validation studies for the use of such markers in quantitative PCR are lacking.

To avoid the problem of copy number variability between species (buffalo and cow), we used the single-copy nuclear gene GH as a reference marker for normalization, whereas a mitochondrial marker (the cyt *b* gene) was selected as the bovine species-specific target, with the aim to achieve the highest sensitivity. Intraspecific as well as intraindividual variation in copy number is minimized through the use of bulk milk that averages individual mtDNA contributions. In addition, inter-specific variation does not occur since a single species (*B. taurus*) is targeted. The variability of mtDNA copy number of milk produced in different periods of the year was checked in order to verify the magnitude of the physiological variation in the functionality of the mammary gland. As reported in **Table 2**, such variability resulted not significant.

Validation experiments, performed on DNA extracted from blood of various Italian milk breeds, showed that no differences in amplification efficiency were present due to sequence polymorphisms across breeds. In addition, no cross-amplification was observed by bovine-specific cyt *b* primers when water buffalo DNA was used as template. Although consistent amplification and species specificity was achieved using SYBR green I as the detection dye, the employment of the TaqMan MGB probe as the detection system markedly increased the efficiency and sensitivity of the assay. The comparison between SYBR green I and the TaqMan MGB probe (**Table 1**) clearly demonstrates that the use of fluorescent probes allows the reliable quantification of DNA extracted from highly processed matrixes like cheese. The SYBR green I assay shows that the efficiency is matrix-linked; considering the same matrix (cheese), the probe assay evidenced a greater efficiency both for reference and target genes; moreover, the similarity observed for these values corroborates the test consistency.

The TaqMan MGB real-time PCR assay reported in the present study showed a very high sensitivity with a limit of detection of 0.1% bovine DNA, while a very high repeatability, checked across a series of independent extractions and amplifications, was recovered (data not shown).

After validation on laboratory samples, the diagnostic test was applied on a wide collection of commercial mozzarella cheeses. The results obtained evidenced that the majority of the PDO Mozzarella di Bufala Campana samples were often contaminated with bovine milk. Only four samples (three different brands, one of them was PDO) have been found to contain bovine milk at a level higher than 5%. The main part of bovine-positive products were sampled during the summer (P, 57.1%; PNQ, 28.6%; N, 14.3%), while in the autumn the situation was different (P, 22.2%; PNQ, 52.8%; N, 25%). During the summer, market demand for buffalo mozzarella goes up, while buffalo milk production could not be adequate to satisfy it. Therefore, a limited addition of bovine milk could represent a compromise between the convenience of using a less expensive and more easily available milk and the risk of loosing the peculiar taste of the product if too large an amount of cow milk is added. Alternatively, the contamination with a limited amount of bovine milk could also be explained as a consequence of using not-well-cleaned equipment machinery in those plants where bovine milk is also processed. Given the observed levels of contamination, such an occurrence would represent a serious case of bad production practice. A third possible explanation is the use of selected whey as starter, instead of natural whey culture, for acidification. Starter whey is dehydrated after being cultivated in bovine milk, and therefore, it is likely that in the centrifugation step for whey separation from milk bovine cells might also be pelleted and included in the preparation of the starter. Analytical evaluation of the standard samples produced with the addition of selected whey allowed us to estimate the realistic amount of bovine DNA coming from such a way. These standard samples evidenced just PNQ results, indicating that contamination is present but at a level lower than 0.6%. Therefore, it cannot be excluded that the PNQ commercial samples were produced using starter whey.

Summarizing, the real-time Q-PCR method tested and presented in this study satisfies the needs of sensitivity, specificity, and efficiency. The quantitative values of cow dairy matter observed in the tested commercial water buffalo mozzarella cannot be considered negligible in the case of allergy disease. Therefore, the assay presented could be conveniently employed to carry out routinely and accurately controls aimed not only to discourage any fraudulent behavior but also to reduce risks for consumer health.

ACKNOWLEDGMENT

We are grateful to Emilio Pastore (Dipartimento di Scienze Animali, Università di Padova, Agripolis, Legnaro, Italy) for animal blood gathering, to Antonio Cattelan (Istituto per la Qualità e le Tecnologie Agroalimentari, Veneto Agricoltura, Thiene, Vicenza, Italy) for his precious advices about cheese making, to Olivia Bodo and Alba Mininni for collaboration in the experimental analysis, and to the cattle and buffalo breeders in Montecchio Precalcino (Vicenza, Italy) and in Piove di Sacco (Padova, Italy) for milk collection.

LITERATURE CITED

(1) Commission Regulation (EC) No. 1107/1996 of the Commission of 12 June Registration of geographical indications and designations of origin under the procedure laid down in Article 17 of Council Regulation (EEC) No. 2081/92. *Official Journal of the European Commission*, June 21, 1996, pp 1−15.

(2) Commission Regulation (EC) No. 213/2001 of the Commission of 9 January Annex XV, Reference method for the detection of cows' milk and caseinate in cheeses from ewes' milk, goats' milk or buffalos' milk or mixtures of ewes' and buffalos' milk. *Official Journal of the European Commission*, February 7, 2001, pp 0001−0099.

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

(4) Andreotti, G.; Trivellone, E.; Lamanna, R.; Di Luccia, A.; Motta, A. Milk identification of different species: ^{13}C-NMR spectroscopy of triacylglycerols from cows and buffaloes milk. *J. Dairy Sci.* **2000**, *83*, 2432−2437.

(5) Enne, G.; Elez, D.; Fondrini, F.; Bonizzi, I.; Feligini, M.; Oleandri, R. 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* **2005**, *1094*, 169−174.

(6) Veloso, A. C. A.; Teixeira, N.; Ferreira, I. 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* **2002**, *967*, 209−218.

(7) Bramanti, E.; Sortino, C.; Onor, M.; Beni, F.; Raspi, G. Separation and determination of denaturated 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* **2003**, *994*, 59−74.

(8) Skarpeid, H. J.; Moe, R. E.; Indhal, U. G. Detection of mechanically recovered meat and head meat from cattle in ground beef mixtures by multivariate analysis of isoelectric focusing protein profiles. *Meat Sci.* **2001**, *57*, 227−234.

(9) Hurley, I. P.; Ireland, H. E.; Coleman, R. C.; Williams, J. H. H. Application of immunological methods for the detection of species adulteration in dairy products. *Int. J. Food Sci. Technol.* **2004**, *39*, 873−878.

(10) Woolfe, M.; Primrose, S. Food forensic: using DNA technology to combat misdescription and fraud. *Trends Biotechnol.* **2004**, *22*, 222−226.

(11) Lockley, A. K.; Bardsley, R. G. DNA-based methods for food authentication. *Trends Food Sci. Technol.* **2000**, *11*, 67−77.

(12) Bottero, M. T.; Civera, T.; Numera, D.; Rosati, S.; Sacchi, P.; Turi, R. M. A multiplex polymerase chain reaction for the identification of cow's, goat's and sheep's milk in dairy products. *Int. Dairy J.* **2003**, *13*, 277−282.

(13) Burgalassi, F.; Cioni, L.; Sbranati, E.; Fancinelli, S.; Fani, R.; Gerace, L.; Lanzilao, I.; Settimelli, M. Identificazione di specie nel settore lattiero caseario con tecniche molecolari. *Ind. Aliment. (Pinerolo, Italy)* **2003**, *42*, 736−741.

(14) Maudet, C.; Taberlet, P. Detection of cows' milk in goats' cheeses inferred from mitochondrial DNA polymorphism. *J. Dairy Res.* **2001**, *68*, 229−235.

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

(16) Walker, J. A.; Hughes, D. A.; Anders, B. A.; Shewale, J.; Sihna, K. S.; Batzer, M. A. Quantitative intra-short interspersed element PCR for species-specific DNA identification. *Anal. Biochem.* **2003**, *316*, 259−269.

(17) Plath, A.; Krause, I.; Einspanier, R. Species identification in dairy products by three different DNA-based methods. *Z. Lebensm.-Unters.-Forsch.* **1997**, *205*, 431−441.

(18) De Pilla, N.; Civera, T.; Bottero, M. T. Valutazione della presenza di latte vaccino in mozzarelle dichiarate di pura bufala. *Ind. Aliment. (Pinerolo, Italy)* **2002**, *41*, 393−397.

(19) Di Pinto, A.; Conversano, M. C.; Forte, V. T.; Novello, L.; Tantillo, G. M. Detection of cow milk in buffalo mozzarella by polymerase chain reaction (PCR) assay. *J. Food Qual.* **2004**, *27*, 428−435.

(20) Mafra, I.; Ferriera, I.; Faria, M. A.; Oliveira, B. P. P. A novel approach to the quantification of bovine milk in ovine cheeses using a duplex polymerase chain reaction method. *J. Agric. Food Chem.* **2004**, *52*, 4943−4947.

(21) Anklam, E.; Gadani, F.; Heinze, P.; Pijnenburg, H.; Van Den Eede, G. Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products. *Eur. Food Res. Technol.* **2002**, *214*, 1, 1438−2385.

(22) Fernandez, S.; Charles-Delobel, C.; Geldreich, A.; Berthier, G.; Boyer, F.; Collonnier, C.; Coué-Philippe, G.; Diolez, A.; Duplan, M.-N.; Kebdani, N.; Romaniuk, M.; Feinberg, M.; Bertheau, Y. Quantification of the 35S promoter in DNA extracts from genetically modified organisms using real-time polymerase chain reaction and specificity assessment on various genetically modified organisms, part I: operating procedure. *J. AOAC Int.* **2005**, *88*, 2, 547−557.

(23) López-Andreo, M.; Lugo, L.; Garrido-Pertierra, A.; Prieto, M. I.; Puyet, A. Identification and quantitation of species in complex DNA mixtures by real-time polymerase chain reaction. *Anal. Biochem.* **2005**, *339*, 73−82.

(24) Sawyer, J.; Wood, C.; Shanahan, D.; Gout, S.; McDowell, D. Real-time PCR for quantitative meat species testing. *Food Control* **2003**, *14*, 579−583.

(25) Rodríguez, M. A.; García, T.; Gonzáles, I.; Asensio, L.; Hernández, P. E.; Martín, R. Quantitation of mule duck in goose foie gras using TaqMan real-time polymerase chain reaction. *J. Agric. Food Chem.* **2004**, *52*, 1478−1483.

(26) Presidenza del Consiglio dei Ministri. Protezione transitoria accordata a livello nazionale all modifica del Disciplinare di produzione della Denominazione di origine del formaggio "mozzarella di bufala campana". Allegato al Decreto del Ministero delle Politiche Agricole e Forestali del 18 settembre (Iscrizione nel "Registro delle denominazioni di origine protette e delle indicazioni geografiche protette" ai sensi del Reg. CE n. 1107/96). *Gazzetta Ufficiale della Repubblica Italiana*, June 11, 2003, 35−37.

(27) Thompson, J. D.; Gibson, T. J.; Plewniak, F.; Jeanmougin, F.; Higgins, D. G. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **1997**, *25*, 4876−4882.

(28) Alonso, A.; Martin, P.; Albarran, C.; Garcia, P.; Garcia, O.; de Simon, L. F.; Garcia-Hirschfeld, J.; Rancho, M.; de la Ruja, C.; Fernandez-Piqueras, J. Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. *Forensic Sci. Int.* **2004**, *139*, 141−149.

(29) Masuyama, M.; Iida, R.; Takatsuka, H.; Yasuda, T.; Matsuki, T. Quantitative change in mitochondrial DNA content in various mouse tissues during aging. *Biochim. Biophys. Acta* **2005**, *1723*, 302−308.

(30) Matsunaga, T.; Shibata, K.; Yamada, J.; Shinmura, Y.; Chikuni, K. Identification of meat species based on the difference of 18S ribosomal RNA genes. *J. Jpn. Soc. Food Sci. Technol.* **1998**, *12*, 719−723.

(31) Mendoza-Romero, L.; Verkaar, E. L. C.; Savelkoul, P. H.; Catsburg, A.; Aarts, H. J. M.; Buntjer, J. B.; Lenstra, J. A. Real-time PCR detection of ruminant DNA. *J. Food Prot.* **2004**, *67*, 3, 550−554.

Received for review December 22, 2006. Revised manuscript received February 23, 2007. Accepted March 7, 2007. This research was funded by the Fondazione CARIVERONA−Ban 2004 in the Scientific and Technological field.

JF0637271