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Abstract

A Real-Time PCR Allelic Discrimination TaqMan assay based on the analysis of one diagnosis position enabling the identification of cows’ and buffalo milk in dairy products was developed. Specific primers and probes were designed on the mitochondrial *cytochrome b* gene. In particular, primers were designed upstream and downstream the chosen diagnosis site in a well conserved region for both *Bos taurus* and *Bubalus bubalis.* Two probes were designed to specifically hybridise to *B. taurus* and *B. bubalis* sequences.

This technique proved to be species-specific when tested on blood and pure milk samples giving an unambiguous result plot of rapid interpretation. The detection threshold, evaluated by means of different dilutions, was 2% of cow milk in buffalo milk.

The test, when applied to cheese samples from the retail trade, enabled the identification of partial or total substitution of cow for buffalo milk. Furthermore, it is relatively rapid, as both species can be detected in one step, with no need for any post-PCR manipulation.

Keywords: Species identification; Real-Time PCR Allelic Discrimination; Mozzarella cheese.

Introduction

Species identification in dairy products has a remarkable importance in food traceability and fraud control. These two aspects are relevant when Protected Designation of Origin (PDO) cheeses are considered which are regulated by the European Union laws having strict production policies. Italy has many PDO dairy products; among these “mozzarella di bufala campana” has a great economic relevance. It must be manufactured using only buffalo milk (EC Regulation No. 1107/96), so any trace of cow’s milk in the product labelled “mozzarella di bufala campana” has to be considered a fraud.

For these reasons, adequate control methods are required to evaluate product authenticity and to detect possible fraud (De La Fuente & Juárez, 2005; Mafra, Roxo, Ferreira, & Oliveira, 2007).

The official control method to detect bovine protein in dairy products is based on isoelectric focusing (IEF) of γ-caseins after plasminolysis (EC Regulation No. 213/2001). However the protein profile of a single species produces a complex banding pattern and even small amounts of protein from other species will often overlap the species-specific bands, so impairing the interpretation (López-Calleja et al., 2007b).

Additional methods currently used are based on the analysis of protein fraction, such as Enzyme Linked ImmunoSorbent Assay (ELISA) (Hurley, Ireland, Coleman, & Williams, 2004; López-Calleja, González et al., 2007). However, these methods cannot always discriminate milk from closely related species such as cow and buffalo, or sheep and goat, and are not applicable to heat-treated matrices (López-Calleja et al., 2005).

Other analytical techniques based on protein analysis for species identification in milk products, such as, High-Performance Liquid Chromatography with ElectroSpray Ionization Mass Spectrometry (HPLC/ESI-MS) (Chen, Chang, Chung, Lee, & Ling, 2004) and Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOFMS) (Cozzolino, Passalacqua, Salemi, & Garozzo, 2002) have been applied. On the other hand, these methods are time consuming and therefore unsuitable for routine analysis (Lockley & Bardsley, 2000; Woolfe & Primrose, 2004).

Other methods rely on the interpretation of triglyceride and fatty acid profiles by NanoElectroSpray Ionisation QqTOF Mass Spectrometry (NanoESI-MS) (Mirabaud, Rolando, & Regert, 2007). Obviously, these methods cannot be used for detecting any adulteration in skimmed milk (Mayer, 2005).

At present, biomolecular techniques are largely applied for species identification in dairy products. They have proved to be reliable, specific, sensitive and fast. Some of them, such multiplex PCR (Bottero, Civera, Anastasio, Turi, & Rosati, 2002; Bottero et al., 2003; Rea et al., 200) and PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) (Branciari, Nijman, Plas, Di Antonio, & Lenstra, 2000; El-Rady & Sayed, 2006) are based on non-fluorescent systems; others, like the Real-Time PCR, are based on fluorescent reactions (López-Calleja et al., 2007a, 2007b; Lopparelli, Cardazzo, Balzan, Giaccone, & Novelli, 2007; Zhang, Fowler, Scott, Lawson, & Slater, 2007).

Both fluorescent and non-fluorescent systems have several advantages: multiplex PCR assay can be used to detect different species by means of a single reaction; PCR-RFLP analysis can be used to identify closely related species and Real-Time PCR does not

require any post amplification step and has a high automation potential, allowing the processing of large numbers of samples.

The aim of the present study was to develop a fluorescent qualitative test, based on the analysis of the diagnosis site by means of a Real-Time PCR Allelic Discrimination TaqMan MinorGroove Binding (MGB) test. This method enables the identification of bovine and buffalo DNA in pure buffalo cheese products by means of a single reaction.

Materials and methods

Samples

Ten buffalo (*Bubalus bubalis*) and 10 bovine (*Bos taurus*) blood samples were used as positive control to set up the assay. Similarly, 10 samples of buffalo milk and 10 samples of cows' milk were tested.

In addition, test sensitivity was evaluated. Some cows' and buffalo milk samples were subjected to somatic cell count (Somacount 150, Bentley Instruments, Chaska, MN, USA) and diluted in Phosphate Buffered Saline (PBS) for normalisation. Successively, dilutions of cows' milk mixtures in buffalo milk (30%, 20%, 10%, 4%, 2%, 0.4%, 0.2%, 0.04%, 0.02% and 0.004%) were prepared.

Cheese samples included forty samples of commercial "mozzarella di bufala campana" PDO, five mozzarella cheeses made with cows' milk, six samples allegedly made only with buffalo milk (no PDO) and one cheese made with both cows' and buffalo milk (mixed mozzarella). Samples were purchased at local supermarkets and dairy shops or directly obtained from cheese manufacturers.

DNA extraction

To extract the DNA from blood, heparinised samples were subjected to centrifugation at 3000*g* for 20 min and 1 ml of buffy coat was withdrawn and stored at −20 °C. DNA was extracted from 50 µl of buffy coat using Dneasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for blood samples.

Milk samples (1 ml) were centrifuged at 1500*g* for 15 min to collect somatic cells. The pellets were rinsed three times in 1 ml of PBS, centrifuged at 12,000*g* for 5 min and finally resuspended in 200 µl of PBS. DNA was extracted following the protocol of Dneasy Blood & Tissue kit (Qiagen, Hilden, Germany).

DNA from cheese samples was extracted following the animal tissue protocol of Dneasy Blood & Tissue kit (Qiagen, Hilden, Germany).

DNA was quantified by spectrophotometry (BioPhotometer 6131, Eppendorf AG, Hamburg, Germany).

Primers and probes design

The *cytochrome b* (*cyt b*) sequences obtained from the GenBank database, corresponding to *B. taurus* (GenBank Accession No. NC006853) and *B. bubalis* (GenBank Accession No. NC006295) were aligned with the ClustalW software (Higgins, Bleasby, & Fuchs, 1992) for detection of the polymorphic site to be used as diagnosis position (Table 1). Twenty sequences available in GenBank for each species were later examined to confirm the absence of intraspecific variation of the chosen diagnosis site.

Based upon these sequences alignment, we designed a TaqMan MGB Allelic Discrimination assay around the polymorphic site using Primer Express software (Applied Biosystems, Foster City, CA, USA).

Forward (5′-GCAAACGGAGCTTCAATGTTTT-3′) and reverse (5′-CGTATCCTATAAATGCTGTGGCTATTACT-3′) primers were designed upstream and downstream the chosen diagnosis site in a well conserved region both in *B. taurus* and in *B. bubalis* (Table 1).

Primers were synthesised by Operon (Cologne, Germany).

One probe was designed to specifically hybridise, with 100% of homology, to the *B. taurus* sequence (5′-6FAM-TTATATATGCACGTAGGACG-3′-MGB) and the other was designed to hybridise, with 100% of homology, to the *B. bubalis* sequence (5′-VIC-TTATATATACACGTAGGACGAG-3′-MGB).

Probes were synthesised by Applied Biosystems (Foster City, CA, USA).

Analysis of diagnosis site

Real-Time PCR was performed in a 25 µl reaction mixture containing 900 nM of both forward and reverse primers, 200 nM of each probe, 12.5 µl TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA) and 10 ng of DNA template. Thermal cycling was performed on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 1 min.

All PCR reactions were run in duplicate.

Results were obtained by the automatic calling feature of the Allelic Discrimination option in SDS v1.2.1 software (Applied Biosystems, Foster City, CA, USA). Species were assigned to samples by visually inspecting a plot of the fluorescence (Rn) from the buffalo probe versus the Rn from the bovine probe generated from the post-PCR fluorescence read.

A duplex PCR assay was used as a confirmatory test (Bottero et al., 2002).

Results

Analysis of the alignment of the reference sequences obtained from GenBank showed that the base in 288 position (G/A) of *cyt b* gene could differentiate between *B. taurus* and *B.*

bubalis (Table 1). This diagnosis site did not show any intraspecific variation when compared with twenty Genbank sequences for each species.

Real-Time PCR Allelic Discrimination TaqMan assay PCR allowed the discrimination of bovine and buffalo DNA in all blood and pure milk samples. A representative plot used to assign the species is displayed in Fig. 1.

After having verified the specificity of the selected primers and probes, experimental milk mixtures were tested in order to determine the assay sensitivity. Amplification reaction was performed on DNA extracted from cow/buffalo raw milk mixtures containing defined percentages of the milk of the two species. The assay consistently revealed the addition of cows' milk to buffalo milk as low as 2% (Fig. 2).

Finally, the Real-Time PCR Allelic Discrimination assay was applied to 52 mozzarella cheeses from the retail trade in order to verify the label statements. Forty-six samples confirmed the information given on the label (forty "mozzarella di bufala campana", five cow mozzarella cheeses and one mixed mozzarella), while six labelled "pure buffalo" (no PDO) did not. In particular, four samples showed the unmistakable presence of cow milk, while in two samples only cow milk was detected (Fig. 3).

All these results were confirmed by duplex PCR.

Discussion

Identification of the animal species in food is gaining increasing importance, because consumers are more and more interested in food origin, also for health reasons.

Strategies employing biomolecular methods for discriminating closely related species involve the use of species-specific primers when the target sequences present an adequate number of mismatches. However, this approach is impaired when there is a high degree of homology, in fact in these cases the analysis of a single nucleotide polymorphism is preferable.

In the last few years, the PCR-RFLP have been widely used for species identification in dairy products (Branciari et al., 2000; El-Rady & Sayed, 2006). PCR-RFLP is based on the analysis of diagnosis sites by means of restriction enzymes.

However, when mixtures of two species are to be detected, the interpretation of PCR-RFLP results could be difficult due to possible overlapping restriction patterns (Bottero et al., 2003).

In addition, the secondary conformation of DNA could make the enzyme access to target site difficult leading to an incomplete DNA digestion. The consequent presence of indigested products causes a misinterpretation of the results (Quinteiro et al., 1998).

Furthermore, the use of many restriction enzymes and the analysis of more than one diagnosis site in order to discriminate numerous species, make PCR-RFLP difficult to automate.

Recently, another method based on the analysis of species-specific mutations has been successfully proposed for the identification of several closely related species such as those belonging to *Thunnus* genus (Bottero, Dalmasso, Cappelletti, Secchi, & Civera, 2007) and some ungulates (La Neve, Civera, Mucci, & Bottero, 2007). The technique described in these papers, based on a multiplex Primer Extension Reaction (PER), enables the detection of the diagnosis site just as precisely as that obtained by sequencing. Nonetheless, the simultaneous presence of two or more species could generate misinterpretation of the results thus limiting its application for food mixture analysis, as observed for PCR-RFLP (Partis et al., 2000).

In our study, we developed a Real-Time PCR Allelic Discrimination TaqMan MGB assay based on a diagnosis site analysis for the identification of cows' and buffalo milk.

Recently, the Real-Time PCR Allelic Discrimination test has been largely used in genetic and microbiological studies in order to identify the polymorphisms responsible for diseases (Stopińska, Grzybowski, Malyarchuk, Derenko, & Miścicka-Śliwka, 2006) and to discriminate between the various species of microorganisms (U'Ren et al., 2005).

During amplification, the fluorogenic TaqMan probes anneal specifically to complementary sequences between forward and reverse primer binding sites on the DNA template and are then degraded owing to the 5′–3′ exonuclease activity of DNA polymerase. Once separated from the quencher, the reporter dye emits fluorescence which is read by the Real-Time PCR system.

In the assay here described, the two probes of bovine and buffalo have 100% homology with their respective target sequences while only a single mismatch in proximity of 5′ end is observed between them. The contemporary presence of the two probes allows the annealing of both regardless of the DNA present (buffalo/bovine), because the single mismatch does not impair the probes' annealing to heterologous DNA. During the extension, the 5′–3′ exonuclease activity of the DNA polymerase degrades the probe that has 100% of homology with the template with a consequent fluorescence emission. On the contrary, the probe having a mismatch with the sequence is removed without degradation and devoid of fluorescence emission. The post-PCR analysis, allows the instantaneous visualisation of the results by means of a specific plot (Fig. 1). The Real-Time Allelic Discrimination test enabled a correct and rapid discrimination of bovine and buffalo DNA extracted from pure milk, cheese and from mixed samples.

Furthermore the application of the method to samples collected from retail trade, proved the authenticity of all the PDO mozzarella samples which resulted to be manufactured only with buffalo milk, following the PDO Regulations (EC Regulation No. 1107/96). In a recent investigation, other authors (Lopparelli et al., 2007) found the presence of cows' milk in several PDO "mozzarella di bufala campana". This result could be ascribed to the high sensitivity (0.1%) of the method used by the above mentioned authors. In fact, even though an high sensitivity has often a great diagnostic value, in this particular context the possibility of detecting very small amounts of cows' milk in dairy products could be a drawback because it could be difficult to distinguish a fraud from an unintentional contamination occurred during processing.

The method described in this paper provides also a competitive advantage over traditional PCR assays, because the system utilises 96-well format plates that can be read in about 5 min (Campsall et al., 2004) so it can be easily used for routine control of dairy products.

Furthermore, the use of fluorescence for DNA analysis eliminates the need for post-PCR processing step, such as gel electrophoresis and ethidium bromide staining (López-Calleja et al., 2007a).

Our results suggest that this Real-Time PCR Allelic Discrimination assay proposed is a reliable way to identify and differentiate between *B. taurus* and *B. bubalis* and represents a potentially valuable diagnostic tool for species identification in “mozzarella di bufala campana”.

In conclusion, this test summarises several advantages: one-step simultaneous detection of two species, timely diagnosis and differentiation of closely related species. Therefore it could be proposed as valid alternative to the PCR-RFLP and to the multiplex PER for species identification in mixed food products.

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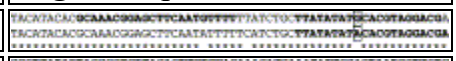
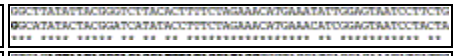
Table 1: Primers and probes design.

Species	GenBank Accession No.	Position	Aligned sequences 5′–3′
B. taurusB. bubalis	NC006853NC006295	241241	
B. taurusB. bubalis	NC006853NC006295	301301	
B. taurusB. bubalis	NC006853NC006295	361361	

Primers binding site for primers and probes (in bold).

The bases in the box correspond to the diagnosis site.

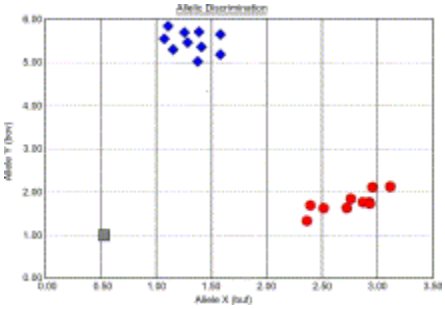


Fig. 1: Real-Time PCR Allelic Discrimination test on blood and milk samples. The plot shows the fluorescence of the buffalo (*x* -axis) and bovine (*y* -axis) probes. In the plot are reported the results of: no template controls (NTC, square), pure bovine samples (diamonds) and pure buffalo samples (circlets).

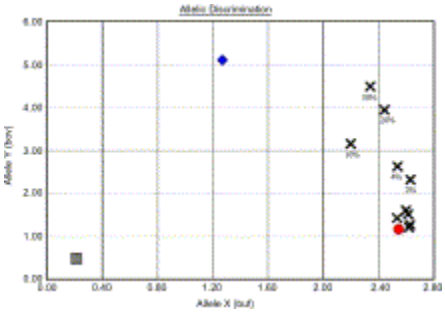


Fig. 2: Real-Time PCR Allelic Discrimination test on dilutions of cows' milk mixtures in buffalo milk. The plot shows the fluorescence of the buffalo (*x* -axis) and bovine (*y* -axis) probes. The plot contains: no template controls (NTC, square), pure bovine milk sample (diamond), pure buffalo milk sample (circlet) and dilutions of bovine/buffalo mixed milk (crosses).

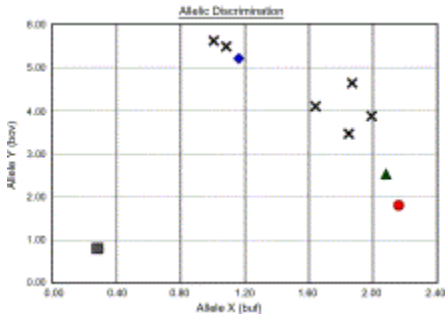


Fig. 3: Real-Time PCR Allelic Discrimination test on samples from the retail trade incorrectly labelled. The plot shows the fluorescence of the buffalo (*x* -axis) and bovine (*y* -axis) probes. The plot contains: no template controls (NTC, square), pure bovine milk sample (diamond), pure buffalo milk sample (circlet), 2% bovine/buffalo milk mix (triangle) and samples labelled “pure buffalo” (no PDO) (crosses).