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Validation of a fast real-time PCR method to detect fraud and mislabeling in milk and dairy products

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ABSTRACT

Fast real-time PCR TaqMan assays were developed and validated for species identification in dairy products. Based on the amplification of *12S rRNA* and *cytB* partial genes of mitochondrial DNA, the methods were demonstrated to be sensitive, fast, and species specific for *Bos taurus*, *Ovis aries*, *Bubalus bubalis*, and *Capra hircus*. The limit of detection calculated was lower than 1%, and the efficiency was reported to be higher than 96% in every assay. An internal amplification control was used to detect possible false negatives. The method was validated by means of laboratory-prepared samples mixing different species. Moreover, 18 commercial dairy samples were analyzed by both real-time PCR and isoelectric focusing, the official EU reference method. The 4 TaqMan assays were confirmed to be a useful tool for milk and dairy product authentication.

Key words: species identification, dairy products, isoelectric focusing, real-time PCR

INTRODUCTION

The European food safety policy aims to protect the customers not only from food pathogens but also from fraudulent species substitutions. Key priorities for these purposes are the correct labeling of food and food traceability and to commission scientific studies if it is necessary to accomplish European Commission Regulation No. 178/2002 (European Commission, 2002). Therefore, innovation in sensitive diagnostic tools is necessary for the authentication of processed food components. Milk and dairy products are an important part of the Mediterranean diet, which includes milk and processed dairy from bovine, sheep, goat, and buffalo origin.

More than a hundred European cheeses of great economic importance are classified as protected designation of origin (**PDO**) or protected geographical indications by European Commission (2012) Regulation No. 1151/2012. A common problem in dairy products is the undeclared substitution of milk with dairy products of lower commercial value because differences in price and seasonal availability make this attractive for farmers and producers. Coupled with that, in terms of food technology, it is more difficult to develop some dairy products (e.g., Mozzarella cheese) from water buffalo milk because the stretching and mechanical spin become a challenge due to the rheological characteristics of buffalo milk casein compared with cow milk casein (Zhang et al., 2007). Moreover, unintentional mislabeling may also occur when several species are handled on the same manufacturing equipment. However, fraudulent or unintentional, such mislabeled products give rise to economic loss and possible dangers to public health because milk proteins from any animals (most commonly bovine) are potential allergens (van Hengel, 2007).

The Commission Regulation (EC) No. 273/2008 of 5 March 2008 lays down detailed rules for the application of Council Regulation (EC) No. 1255/1999 (European Commission, 2008) regarding methods for the analysis and quality evaluation of milk and milk products. This regulation considers the legal limit of milk substitution as 0.99%, and alimentary fraud is defined when a value is equal or higher than 1%. Moreover, the regulation defines the isoelectric focusing (**IEF**) of γ-caseins as the official method for species identification.

Isoelectric focusing is a qualitative method that has proven to be sensitive and accurate for the detection of cow milk in mixed samples, but it shows several limitations: it is not a high-throughput method, it is not quantitative, and the analysis is time consuming. Moreover, the method cannot discriminate goat-sheep mixtures (Addeo et al., 1990; Mayer et al., 1997) and there may be equivocal interpretation of the IEF profile (López-Calleja et al., 2007b). Furthermore, IEF is not applicable for products made by soy milk because some weak interfering bands have been observed. The same drawback was observed for the alternative protein-based method SDS-PAGE. Therefore, neither method is useful for the detection of cow milk in soy milk products (Mayer et al., 2012).

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Other methods were used for species discrimination in dairy products based on the analysis of protein fraction: ELISA (López-Calleja et al., 2007c), HPLC (Mayer, 2005), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Cozzolino et al., 2002). However, at the moment DNA techniques are largely applied for species identification because they have proven to be reliable, specific, sensitive, and fast. In particular, real-time PCR does not require any postamplification step and can be easily automated allowing the analysis of large numbers of samples (López-Calleja et al., 2007a,b; Dalmasso et al., 2011; Cottenet et al., 2011; Rentsch et al., 2013; Iwobi et al., 2015), and it permits quantitative or semiquantitative analysis.

The aim of the present study was to develop and validate 4 real-time PCR TaqMan assays based on the analysis of mitochondrial DNA (**mtDNA**) for species identification of *Bos taurus*, *Bubalus bubalis*, *Ovis aries*, and *Capra hircus* in milk and dairy products. These methods were validated by using laboratory prepared samples. Moreover, 18 commercial milks and cheeses were analyzed comparing the real-time PCR results with those obtained by IEF, whereas 3 soy milk samples were analyzed by real-time PCR only.

MATERIALS AND METHODS

Sample Preparation

Bovine, buffalo, sheep, and goat milks, purchased straight from the farms, were used as reference materials. Binary mixtures were prepared by combining appropriate vol/vol quantities to obtain 1% of each single species in 99% of the other 3 species considered in this study. The DNA was isolated from 200 μL of milk by using the Maxwell 16 Tissue DNA Purification kit (Promega, Madison, WI) according to the manufacturer's instructions. Moreover, 8 bovine whole milks, a PDO Parmigiano Reggiano bovine cheese, a Caciotta mista mixed fresh bovine/ovine cheese, a fresh cheese made with goat milk, a ripened goat cheese, a PDO Pecorino ovine cheese, and 5 buffalo fresh cheeses (3 PDO Mozzarella di Bufala Campana and 2 non-PDO Mozzarella di Bufala) were purchased from local retailers and analyzed by both real-time PCR and IEF. Three soy milk samples were also recovered from dedicated retailers and analyzed by real-time PCR.

The DNA was then extracted from 25 mg of cheese or 200 μL of milk as described above. Then, DNA was quantified by Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) following the provided protocol and diluted up to 1 ng/μL.

Development of Real-Time PCR Assays. The mtDNA sequences from *B. taurus* (DQ186214), *O. aries* (DQ903212), *B. bubalis* (AF547270), and *C. hircus* (AJ885199) were aligned using the Lasergene (DNAStar) software. Mismatches on *12S rRNA* gene sequences were selected to design species-specific primers and probes for buffalo (12S buffalo forward GTAACCTATGAAATGGGAAGAAATGG; 12S buffalo reverse TTACTGCTAAATCCTCCTTTGGTTATTAAT; 12S buffalo probe 6FAM-TACACCAAGAACACCCAAC-MGBNFQ) and goat (12S goat forward TAGGTCAAGGTGTAACCCATGGAA; 12S goat reverse ACTAAATCCTCCTTTGGTCATTAATTTCA; 12S goat probe 6FAM-CTTAAGAAAATTAATACGAAAGCC-MGBNFQ), whereas bovine and ovine sets were previously described (Cammà et al., 2012). In silico specificity was verified by Basic Local Alignment Search Tool analysis. Probes were labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) on the 5′ end and with the minor groove binder-nonfluorescent quencher (MGB-NFQ) on the 3′ end. The melting temperature, GC contents, and secondary structures (hairpin, self-dimers, and cross-dimers) of each primer and probe set were verified by the Primer Express 3.0 test tool (Applied Biosystems, Waltham, MA) and their concentrations were optimized.

Development of an Internal Amplification Control. The internal amplification controls (**IAC**) were developed for each assay as previously described (Cammà et al., 2012). The IAC was designed as a nontarget chimeric DNA fragment containing a portion of the acetyl-coenzyme A carboxylase gene (**ACC**) from turnip flanked by a portion of *cytB* or *12S rRNA* mtDNA sequence complementary to the primers. In each reaction tube, the IAC DNA was co-amplified with the target DNA using the same primers as for the test reaction. A C_t value around 29 was produced by 0.1 fg of the bovine and buffalo IAC DNA, 0.3 fg of the ovine IAC DNA, and 0.1 fg of the caprine IAC DNA.

Real-Time PCR Protocol. The real-time PCR was performed on the 7900HT Fast Real Time PCR System (Applied Biosystems) and analyzed by the software SDS 2.4 (Applied Biosystems). The primer and probe concentration, the DNA quantity for each reaction and the number of amplification cycles were carefully optimized as follows: the 20 μL reaction mixtures contained 1× TaqMan Fast Universal PCR Master Mix, 300 n*M* of specific MGB probe, 300 n*M* of ACC probe, 900 n*M* of specific oligonucleotide primers, nuclease-free water, 5 μL of 1 ng/μL DNA, and 1 μL of IAC DNA.

The reaction protocol used was 20 s at 95°C followed by 35 cycles of 1 s at 95°C and 20 s at 60°C. Fluorescence readings were taken every cycle, and the logarithm of the increment in normalized fluorescence was plotted versus the numbers of cycles. The threshold level was fixed at the same middle exponential position