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PCR detection of cows' milk in water buffalo milk and mozzarella cheese

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Abstract

Polymerase chain reaction (PCR) has been applied for the specific detection of cows' DNA in water buffalo milk and mozzarella cheese by using primers targeting the mitochondrial 12S ribosomal RNA gene. The use of specific primers for cow yielded a 346 bp fragment from cows' milk DNA, whereas no amplification signal was obtained in sheep's, goats' and water buffalo's milk DNA. Analysis of both buffalo milk and buffalo mozzarella cheese mixtures containing different percentages of cows' milk or bovine mozzarella cheese, enabled the specific detection of cow's DNA with a sensitivity threshold of 0.1%. The proposed PCR assay represents a rapid and straightforward method for the detection of adulterations in water buffalo milk and mozzarella cheese.

Keywords: PCR; Cow; Water buffalo; Mozzarella cheese; 12S rRNA gene

1. Introduction

Species identification of animal products has become an important issue regarding the assessment of food composition, and the provision of accurate consumer information. Protection against species substitution or admixture in dairy products is of significant importance for reasons relating to religion, public health and government regulations. The description and labelling of food must be accurate so that consumers can make informed choices about their diet and the products they buy (Herman, 2001; Hernández, et al., 2003).

In response to the increasing demand for transparency in food trade, European laws require producers to state the type of milk used for manufacturing cheese or other milk products. In the dairy sector, the fraudulent misdescription of food contents on product labels has been reported, especially with high added value milk products commanding a premium price (Woolfe & Primrose, 2004).

Mozzarella cheese, a typical Italian product that is marketed worldwide, was originally made from water buffalo (*Bubalus bubalus*) milk. However, in Italy and in other countries, similar products are now made using less expensive bovine milk. Evaluation of the species of origin of the milk used in these products is important, particularly in mozzarella *di bufala campana*, which is a high grade Italian cheese registered by European law with the Protected Designation of Origin (PDO) that can only be made from water buffalo milk (Rea et al., 2001). Nevertheless, bovine milk or mixtures of bovine and water buffalo milk can also be used to make mozzarella that is not protected by the PDO. In these cases, it is necessary to specify on the labels the species used in the manufacture of cheeses (Bottero, Civera, Anastasio, Turi, & Rosati, 2002; Branciari, Nijman, Plas, Di Antonio, & Lenstra, 2000).

Different methods are currently used for species identification in milk and dairy products, which include immunological, electrophoretic, and chromatographic

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techniques. Among these, capillary electrophoresis, two-dimensional electrophoresis, isoelectric focusing of milk caseins, which is the European Community reference method for cows' milk detection (EC Regulation, 1996), HPLC and ELISA are widely reported (De Noni, Tirelli, & Masotti, 1996; Mimmo & Pagani, 1998; Molina, Martín-Álvarez, & Ramos, 1999). However, immunological and electrophoretic methods cannot always distinguish milk from closely related species such as taurine cattle and water buffalo, or sheep and goats, and are often not suitable for detection of heat-treated material. Additionally, chromatographic methods detect differences in the percentages of the fatty acids but result rather laborious.

In recent years, molecular techniques have been applied to species differentiation and have proved to be reliable, sensitive and fast. Among these methods, PCR is the most widely used molecular technique for the identification of the species of origin in food (Dalmasso et al., 2004; Herman, 2001). However, application of PCR-based techniques for the authentication of dairy products has been less used in comparision to other foods such as meat or fish (Asensio et al., 2001; Matsunaga et al., 1999).

Recent studies have shown that it is possible to use milk as a source of DNA and as a substrate for PCR. Ruminant milk from healthy mammary glands has a large amount of somatic cells, predominantly leukocytes but also epithelial cells, which contain genomic DNA suitable for PCR amplification (Amills, Francino, Jansa, & Sánchez, 1997; Lipkin, Shalom, Khatib, Soller, & Friedmann, 1993; Murphy, Reza Shariflou, & Moran, 2002). Although mammary somatic cells survive during cheese manufacturing and maturation, only a few methods for the isolation of genomic DNA after cheese-making and its use as a template for PCR amplification have been reported (Bottero et al., 2002; Bottero et al., 2003; Branciari et al., 2000; Calvo, Osta, & Zaragoza, 2002; Maudet & Taberlet, 2001; Rea et al., 2001).

In this paper, we describe the development of a specific PCR method for detecting the presence of cows' milk in both buffalo milk and buffalo mozzarella cheese. The assay is based on the amplification of species-specific fragments on mitochondrial DNA (mtDNA) 12S rRNA gene using cow-specific primers and provides a specific, sensitive and effective alternative to other existing methods.

2. Materials and methods

2.1. Milk samples

Several batches of authentic raw milk from cow (*Bos taurus*), sheep (*Ovis aries*) goat (*Capra hircus*) and water buffalo (*Bubalus bubalus*) were obtained from local dairy farms. Samples were transported to the laboratory under refrigeration and were processed immediately or stored frozen at −85 °C until used.

Five independent series of binary mixtures of cows' milk in water buffalo milk were prepared for further DNA extraction and PCR analysis. For each series, six different bovine milk percentages containing 0.1%, 0.5%, 1%, 5%, 10% and 100% (v/v) were prepared in a final volume of 1 mL. Raw, pasteurized (65 °C, 30 min) and sterilized (121 °C, 20 min) milk mixtures were included in the analyses.

Somatic cell counts were performed on five different batches of pure cows' milk, including raw, pasteurized and sterilized samples. These analyses were carried out in an accredited laboratory (LILAasturias, Laboratorio Interprofesional Lechero y Agroalimentario de Asturias, Spain) using a Foss-O-Matic 5000 (Foss Electric, Denmark).

2.2. Cheese samples

Different commercial brands of Mozzarella di bufala campana (DOP) and bovine mozzarella cheese samples were purchased from several local supermarkets. Five independent series of binary cheese mixtures of buffalo mozzarella containing 0.1%, 0.5%, 1%, 5%, 10% and 100% (w/w) of bovine mozzarella cheese were prepared in a final weight of 250 g using a blender (Sunbeam Oster, Florida, USA). Two-gram aliquots of each homogenate were used for further DNA extraction and PCR analysis.

2.3. DNA extraction from milk and cheese

Total cellular DNA was extracted from milk mixtures (raw and heat-treated) using a Wizard DNA cleanup kit (Promega, Madison, WI). Somatic cells were recovered previously from milk by the use of a milk clearing solution (0.15 M *N*-[2-acetamido]-2-iminodiacetic acid (ADA) (Sigma-Aldrich GmbH, Steinheim, Germany), 0.5% w/v triton X-100 (Sigma-Aldrich) and 0.01% w/v uniform polystyrene particles (Bangs Laboratories, Inc., Fishers, size 0.787).

The extraction procedure was performed as follows: 0.5 mL of the clearing solution was added to 1 mL of the milk sample. Samples were mixed by inverting them 10 times before they were centrifuged at $15{,}000 \times g$ in a microcentrifuge for 5 min. The result was a cream pad on top of a clear supernatant. Both the pad and the supernatant were carefully removed, while the pellet left at the bottom of the tube was resuspended in 860 μL of extraction buffer, pH 8.0 (10 mM Tris, 150 mM NaCl, 2 mM EDTA, and 1% SDS), 100 μL of 5 M guanidine hydrochloride, and 40 μL of 20 mg mL^{-1} proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany).