Application of Polymerase Chain Reaction to Detect Adulteration of Sheep's Milk with Goats' Milk

I. López-Calleja, I. González, V. Fajardo, I. Martín, P. E. Hernández, T. García, and R. Martín

Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

ABSTRACT

The polymerase chain reaction has been applied for the specific detection of goats' milk in sheep's milk using primers targeting the mitochondrial 12S ribosomal RNA gene. The use of goat-specific primers yielded a 122-bp fragment from goats' milk DNA, whereas no amplification signal was obtained in sheep's, cows', and water buffaloes' milk DNA. Polymerase chain reaction analysis of raw and heat-treated milk binary mixtures of sheep/goat enabled the specific detection of goats' milk with a sensitivity threshold of 0.1%. This study demonstrates the usefulness of the proposed polymerase chain reaction assay for authentication of milk products in routine analysis.

(Key words: polymerase chain reaction, goats' milk, sheep's milk, 12S rRNA gene)

INTRODUCTION

In recent years, species identification of animal products has become an important issue with regard to food authentication (Sawyer et al., 2003). Particularly, protection against species substitution or admixture in dairy products is important for reasons related to allergic and religious restrictions and to government regulations (Branciarri et al., 2000). For instance, because of its lower cost, undeclared caprine milk is often added in the manufacture of cheeses that are misleadingly labeled with the names of more expensive milk-producing species, such as "pure sheep" (Calvo et al., 2002). Thus, to avoid unfair competition and assure an honest labeling policy, it is necessary to develop good analytical procedures to be used by the dairy industry and law enforcement agencies in an attempt to fight adulteration of milks destined to cheese manufacture.

Numerous analytical methods such as immunological, electrophoretic, and chromatographic techniques

have been developed for species identification of milk and dairy products. Capillary electrophoresis (Molina et al., 1999), 2-D electrophoresis (Chianese et al., 1990), isoelectric focusing of milk caseins (Addeo et al., 1990; Moio et al., 1990), and HPLC (De Noni et al., 1996) are worth mentioning. Among immunological methods, ELISA has been used extensively for dairy product authentication because it is easy to use, rapid, and readily automated (Haza et al., 1997; Richter et al., 1997; Hurley et al., 2004a,b). Although all of these techniques are effective and widely used, immunological and electrophoretic methods are often not suitable for analysis of food products with complex matrices and are significantly less sensitive in heat-treated material. Additionally, chromatographic techniques detect differences in the percentages of the fatty acids but result rather laborious (Branciarri et al., 2000).

In the past decade, DNA has replaced protein in species identification methods due to its stability at high temperatures and to the fact that its structure is conserved within all cells of an individual. The presence of species-specific DNA sequences in animal tissues and the possibility of detecting such sequences specifically has resulted in the emergence of assays based on DNA hybridization probes, PCR, or RFLP applied to the authentication of food (Ebbehøj and Thomsen, 1991; Russell et al., 2000; Wang et al., 2000; Sun and Lin, 2003). Among these, PCR is the most widely used technique for the identification of the species of origin in food (Rodríguez et al., 2002; Dalmasso et al., 2003). However, so far, the application of PCR-based techniques to the authentication of milk and dairy products has been less reported than to other food matrices such as meat or fish (Plath et al., 1997; Bania et al., 2001; Rea et al., 2001; Bottero et al., 2002, 2003; López-Calleja et al., 2004; Mafra et al., 2004).

Ruminant milk contains a large number of somatic cells, which can be used as a convenient source of DNA. Several studies have shown that cellular milk DNA can be consistently amplified by PCR to discriminate species (Lipkin et al., 1993; Amills et al., 1997; Maudet and Taberlet, 2001). This paper describes the development of a rapid PCR-based technique for the detection

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Corresponding author: Isabel González Alonso; e-mail: gonzalzi@vet.ucm.es.

of goats' milk in sheep's milk by targeting the 12S rRNA gene. The proposed assay relies on purification of cellular DNA from milk, followed by mitochondrial DNA amplification with goat-specific primers, and visualization of amplicons on agarose gels. The PCR method developed in this work represents a rapid, sensitive, and highly reliable method for the routine quality control of dairy products.

MATERIALS AND METHODS

Selection and Preparation of Samples

Authentic samples of pooled raw milk from goat (*Capra hircus*), sheep (*Ovis aries*), cow (*Bos taurus*), and water buffalo (*Bubalus bubalus*) were obtained from the collection tank of a local dairy farm. Samples were transported to the laboratory under refrigeration and were processed immediately or stored frozen at -85°C until used.

Binary milk mixtures of goats' milk in sheep's milk were prepared for further DNA extraction and PCR analysis. On these mixtures, 6 different caprine milk percentages containing 0.1, 0.5, 1, 5, 10, and 100% (vol/vol) 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 analysis.

DNA Extraction

DNA extraction from milk samples was performed as previously described (López-Calleja et al., 2004).

Design of Goat-Specific Primers and Amplification of Selected DNA Fragments from Milk Samples

Amplification, purification, and sequencing of a conserved 12S rRNA gene fragment (720 bp) from goats', sheep's, cows', and water buffaloes' milk was accomplished following the procedure described by López-Calleja et al. (2004).

The data obtained after alignment of goat, sheep, cow, and water buffalo 12S rRNA gene sequences (GenBank accession no. AJ849535, AJ849534, AJ849533, and AJ846850, respectively) were used to design the following primer pair, potentially suitable for the amplification of a goat-specific 122-bp DNA fragment: *12SCH-DIR* (5'-AAACGTGTAAAGCACTACATC-3') and *12SCH-INV* (5'-GTCTTAGCTATAGTGTATCAGCTGCA-3').

A sheep-specific primer pair: *12SM-FW* (5'-CTAGAGGAGCCTGTTCTATAATCGATAA-3') and *12SOA-INV* (5'-GTCTCCTCTCGTGTGGTTGAGATA-3') was also designed to verify the presence of sheep's DNA in milk samples in a parallel PCR control assay. This primer

set was expected to yield a 371-bp fragment from sheep's DNA. The specificity of the primer sets was confirmed by challenging them with milk samples of different breeds of goat ("Murciana", "Majorera", and "Palmera") and sheep ("Assaf", "Merina", "Manchega", "Lacha", "Churra", "Castellana", and "Carranzana") in preliminary PCR experiments.

Once the suitability of the primers was assessed, raw and heat-treated binary mixtures of sheep's milk containing different amounts of goats' milk were tested for DNA amplification with goat-specific primers.

Double-stranded amplifications were carried out in a final volume of 50 μL containing 100 ng of template DNA, 2mM MgCl_2 , 10 pmol of each primer, 200 μM of each dNTP, and 2 U of Tth DNA polymerase (Biotools, Madrid, Spain) in a reaction buffer containing 75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM $(\text{NH}_4)_2\text{SO}_4$, and 0.001% BSA. Amplification was carried out in a Progene Thermal Cycler (Techne Ltd., Cambridge, UK), programmed to perform a denaturation step of 93°C for 2 min, followed by 40 amplification cycles which were performed with the following step-cycle profile: strand denaturation at 93°C for 30 s, primer annealing at 63°C for 30 s, and primer extension at 72°C for 45 s. The last extension step was 5 min longer.

The detection limit of the method was estimated by agarose gel electrophoresis of the PCR products obtained from each milk binary mixture containing percentages of goats' milk ranging from 0.1 to 100% (vol/vol).

RESULTS

A PCR-based method has been developed for the specific detection of goats' milk in sheep's milk. After sequencing and alignment of the 12S rRNA conserved fragments obtained from goat (AJ849535), sheep (AJ849534), cow (AJ849533), and water buffalo (AJ846850) (Figure 1), a primer pair specific for goat (*12SCH-DIR*—*12SCH-INV*) was designed complementary to gene fragments of the 12S rRNA in which differences with the other species were of importance. This primer set was expected to yield a goat-specific amplicon of 122 bp in the 12S rRNA gene.

As can be seen in Figure 2, DNA extracted from goats' milk was successfully amplified with *12SCH-DIR*—*12SCH-INV* primer pair, giving rise to the expected PCR fragment, whereas no amplification products were obtained with DNA from sheep, cow, and water buffalo milk samples. To confirm the presence of sheep's DNA when no bands are visible in the gel with goat-specific primers, milk samples were also analyzed with the sheep-specific primer set *12SM-FW*—*12SOA-INV*. As can be seen in Figure 3, sheep's DNA was successfully

Figure 1. Electrophoretic analysis of the 12S rRNA PCR products obtained from goats' (lane 1), sheep's (lane 2), cows' (lane 3), and water buffaloes' (lane 4) milk DNA using primers *12S-FW* and *12S-REV*. NC = Negative control; M = molecular weight marker 1 kb plus DNA ladder (GibcoBRL, Barcelona, Spain).

Figure 2. Electrophoretic analysis of the goat-specific 12S rRNA amplification from milk samples using primers *12SCH-DIR* and *12SCH-INV*. Samples are: goat (lane 1), sheep (lane 2), cow (lane 3), and water buffalo (lane 4). CN = Negative control; M = molecular weight marker 1 kb plus DNA ladder (GibcoBRL, Barcelona, Spain).

amplified with this primer pair yielding the expected 371-bp PCR fragment, whereas no amplification bands were detected for the other species tested.

To determine the detection limit of the PCR, amplification reactions were first performed on DNA extracted from binary raw milk mixtures (goat in sheep) comprising 0.1, 0.5, 1, 5, 10, and 100% (vol/vol) of goats' milk. Figure 4 shows the goat-specific 122-bp band obtained from the goat/sheep milk mixtures, along with the relationship between template DNA amounts and band intensity. The detection threshold was 0.1%.

Milk mixtures of goat/sheep were also subjected to heat-treatments of pasteurization (65°C, 30 min) and sterilization (121°C, 20 min) before being tested for PCR amplification under the conditions described. Similar amplification patterns and detection limits to those obtained for raw milk samples were generated (Figures 5A and 5B).

DISCUSSION

During the last few years, numerous methods based on DNA analysis have been used to monitor adulterations of food products of animal origin (Partis et al., 2000). The most extended techniques involve PCR am-

plification of a conserved gene fragment from a group of species by using universal primers, or amplification of DNA with specific primers for identification of a defined target organism (Bania et al., 2001; Colgan et al., 2001). This paper presents a PCR assay for the detection of goats' milk in sheep's milk using goat-specific primers targeting the mitochondrial 12S rRNA gene. This marker was selected because of its variability, the availability of mitochondrial sequence data from many vertebrates, and the high copy number of mitochondrial DNA relative to other genes (i.e., nuclear DNA).

Many factors are known to influence successful amplification of target sequences by PCR. However, accurate identification of species by PCR is predominantly determined by the specificity of the primers used, which should hybridize to DNA segments with a sufficient degree of species-to-species mismatch. In this context, specific primers have been recently developed for detection of the animal origin of milk products by means of simplex or multiplex PCR formats (Branciari et al., 2000; Rea et al., 2001; Bottero et al., 2002, 2003; López-Calleja et al., 2004).

To make the detection of goats' milk DNA unequivocal, a primer set specific for goat was designed following sequence alignment and comparison of 12S rRNA con-

Figure 3. Electrophoretic analysis of the sheep-specific 12S rRNA amplification from milk samples using primers *12SM-FW* and *12SOA-INV*. Samples are: goat (lane 1), sheep (lane 2), cow (lane 3), and water buffalo (lane 4). NC = Negative control; M = molecular weight marker 1 kb plus DNA ladder (GibcoBRL, Barcelona, Spain).

served fragments amplified from goats', sheep's, cows', and water buffaloes' milk. As expected from sequence analysis, the primer pair *12SCH-DIR*—*12SCH-INV* amplified a 122-bp fragment from goats' DNA, whereas no amplification bands were detected in sheep, cow, and water buffalo species (Figure 2).

Besides the need for suitable primers, adequate protocols for extraction of inhibitor-free DNA are still necessary to achieve consistent PCR amplification of a specific DNA sequence, even from complex or highly processed matrices such as milk or cheese products (Calvo et al., 2002; Murphy et al., 2002; Mafra et al., 2004). In this work, the extraction of inhibitor-free DNA from milk samples, capable of being optimally amplified by PCR, was accomplished using a milk-clearing solution followed by a DNA extraction protocol based on a commercial kit, as described by López-Calleja et al. (2004).

The sensitivity of the assay for identification of goats' milk in sheep's milk was also evaluated as a part of this study. For this purpose, PCR amplification was performed on binary raw mixtures of goat/sheep comprising 0.1, 0.5, 1, 5, 10, and 100% (vol/vol) of goats' milk. It was observed that the lower the percentage of goats' milk in the admixture, the fainter the band obtained in the PCR with the goat-specific primer pair.

Figure 4. Electrophoretic analysis of the 12S rRNA PCR products obtained from raw milk binary mixtures of goat in sheep using primers *12SCH-DIR* and *12SCH-INV*. Samples are: goat 100% (lane 1), goat 10% (lane 2), goat 5% (lane 3), goat 1% (lane 4), goat 0.5% (lane 5), goat 0.1% (lane 6), and sheep 100% (lane 7). NC = Negative control; M = molecular weight marker 1 kb plus DNA ladder (GibcoBRL, Barcelona, Spain).

The detection limit (lowest milk percentage yielding visible DNA amplification) of the assay was 0.1% of goats' milk in the goat/sheep binary milk mixtures (Figure 4). Previously reported PCR-based protocols for dairy product authentication, and particularly for species identification in cheese, exhibit lower or similar sensitivity. For example, Bottero et al. (2003) reported a 0.5% detection limit with a multiplex PCR which identified, in a single reaction step, the milk of bovine, ovine, and caprine species in dairy products; Mafra et al. (2004) and Maudet and Taberlet (2001) reported 0.1% detection of bovine milk in ovine or caprine cheeses with simplex or duplex PCR formats; and Rea et al. (2001) reported a detection limit of 1% using a duplex PCR for the identification of bovine and water buffalo milk used in the manufacture of Mozzarella cheese.

One of the clear advantages of PCR-based methods over other techniques is the combination of a high degree of specificity and the ability to perform reliably with highly processed samples. Although DNA, like protein, undergoes denaturation at elevated temperature and other food processing treatments, it has been observed that DNA can still be detected by short fragment

Figure 5. Electrophoretic analysis of the 12S rRNA PCR products obtained from heat-treated milk binary mixtures of goat in sheep using primers *12SCH-DIR* and *12SCH-INV*. A) Pasteurized samples (65°C, 30 min); and B) sterilized samples (120°C, 20 min). Lanes are: goat 100% (lane 1), goat 10% (lane 2), goat 5% (lane 3), goat 1% (lane 4), goat 0.5% (lane 5), goat 0.1% (lane 6), and sheep 100% (lane 7). NC = Negative control; M = molecular weight marker 1 kb plus DNA ladder (GibcoBRL, Barcelona, Spain).

amplification (Janssen et al., 1998; Behrens et al., 1999; Colgan et al., 2001; Dalmasso et al., 2004). In this work, we studied the effect of thermal treatment of milk on the technique's ability to detect species, through the analysis of experimentally pasteurized and sterilized binary milk mixtures. The results obtained with heat-treated samples showed that the banding pattern and lower detection limit of goats' milk in the PCR developed were not substantially modified with respect to raw milk mixtures (Figures 5A and 5B).

It can be concluded that the PCR method described in this paper is specific and sensitive, providing a low detection limit of goats' milk substitution (0.1%) in milks of a higher commercial value. This PCR assay may therefore be appropriate for food inspection services to detect fraudulent manipulations such as the use of cheaper caprine milk in the manufacture of cheeses supposedly derived from pure sheep's milk; thereby protecting traditional manufacturers and consumers against food product adulteration and misrepresentation.

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