Rapid Detection of Cows' Milk in Sheeps' and Goats' Milk by a Species-Specific Polymerase Chain Reaction Technique

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ABSTRACT

A polymerase chain reaction (PCR) assay was developed for the specific identification of cows' milk in sheep's and goats' milk by using primers targeting the mitochondrial 12S rRNA gene. The use of a forward primer complementary to a conserved DNA sequence, along with a reverse primer specific for cow, yielded a 223-bp fragment from cows' milk DNA, whereas no amplification signal was obtained in sheep's and goats' milk DNA. The technique was applied to raw, pasteurized, and sterilized milk binary mixtures of cow-sheep and cow-goat, enabling the specific detection of cows' milk with a good sensitivity threshold (0.1%). The proposed PCR assay represents a rapid and straightforward method applicable to the authentication of milk and other dairy products in routine analysis.

(Key words: polymerase chain reaction, cow milk, sheep and goat milk, 12S ribosomal ribonucleic acid gene)

INTRODUCTION

Species identification in animal products has received great attention in recent years. Particularly, species identification of dairy products has a remarkable importance for several reasons, including frequent human adverse reactions toward some species milk proteins and government regulations (Bottero et al., 2003). In many European countries, laws require producers to state the type of milk used for manufacturing cheese or other milk products. However, a common fraudulent practice found in the dairy production line is the use of a less costly type of milk in substitution of more expensive ones. An outstanding example is the addition of cows' milk in the manufacture of cheeses that are falsely labeled with the names of more expensive milk-

producing species, like "pure sheep," "pure goat," or mixtures of sheep and goat (Calvo et al., 2002). To avoid unfair competition and to assure consumers of accurate labeling, it is necessary to develop methods for assessing if the species or the percentage of milk in a milk mixture corresponds to the legal requirement.

So far, many different analytical approaches, such as immunological, electrophoretic, and chromatographic techniques, have been developed for species identification of milk and dairy products. Among these, capillary electrophoresis (Molina et al., 1999); 2-dimensional electrophoresis (Chianese et al., 1990); isoelectric focusing of milk caseins (Addeo et al., 1990; Moio et al., 1990), which is the European Community reference method for detection of cows' milk in non-bovine dairy products (EC regulation no. 1081/96); HPLC (De Noni et al., 1996); and ELISA (Anguita et al., 1996; Haza et al., 1997) are worth mentioning. However, immunological and electrophoretic methods are often not suitable for food products with complex matrices, being also significantly less sensitive in heat-treated material. Chromatographic methods detect differences in the percentages of the fatty acids, but the method is rather laborious (Branciari et al., 2000).

More recently, genetic techniques have been applied for species differentiation and have proved to be reliable, sensitive, and fast. Today, PCR is the most widely used genetic technique for the identification of the species of origin in food, especially in meat products (Chikuni et al., 1994; Koh et al., 1998; Matsunaga et al., 1999; Rodríguez et al., 2003). In contrast, application of PCR-based techniques to the authentication of dairy products has been very limited.

Milk from healthy mammary glands contains a large number of somatic cells (leukocytes and epithelial mammary cells). Recent studies have shown that it is possible to use these cells as a source of DNA, which can be consistently amplified by PCR to discriminate species (Lipkin et al., 1993; Amills et al., 1997; Maudet and Taberlet, 2001). The aim of the present study was to develop a rapid PCR-based method for the detection of cows' milk in sheep's and goats' milk mixtures. The

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assay is based on the amplification of a cow-specific fragment on mtDNA of the 12S rRNA gene and the visualization of PCR products on agarose gels. This work may provide a specific, sensitive, and inexpensive alternative to other existing methods.

MATERIALS AND METHODS

Selection and Preparation of the Samples

Authentic samples of pooled raw milk from cow (*Bos taurus*), sheep (*Ovis aries*), and goat (*Capra hircus*) were obtained from a 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.

Two series of binary milk mixtures of cows' milk in sheep's or goats' milk were prepared for further DNA extraction and PCR analysis. For each series, 6 different cow milk percentages containing 0.1, 0.5, 1, 5, 10, and 100% (vol/vol) were prepared in a final volume of 1 mL. Samples analyzed included raw, pasteurized (65°C , 30 min), and sterilized (121°C , 20 min) milk mixtures.

DNA Extraction

Total cellular DNA was extracted from raw and heat-treated milk mixtures using a Wizard DNA cleanup kit (Promega, Madison, WI). Previously, somatic cells were recovered 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% wt/vol Triton X-100 (Sigma-Aldrich), and 0.01% wt/vol uniform polystyrene particles (size 0.787; Bangs Laboratories, Inc., Fishers, IN).

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 the top of a clear supernatant. Both the pad and the supernatant were carefully removed, and 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 proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany). The samples were incubated overnight at 55°C with shaking at 60 rpm (C24KC; New Brunswick Scientific Co., Edison, NJ), and they were left to cool at room temperature. Five hundred microliters of chloroform (Sigma-Aldrich) were added to the lysate before centrifugation at 13,000 rpm for 10 min. The clear aqueous supernatant obtained after the centrifugation (500 μL) was used to purify the DNA using the Wizard DNA

cleanup system kit (Promega) with a vacuum manifold, according to the manufacturer's instructions. Finally, the DNA was eluted in 100 μL of sterile deionized water, and its concentration was determined by spectrophotometry at 260 nm.

PCR Amplification of a Conserved 12S rRNA Gene Fragment from Cows', Sheeps', and Goats' Milk

Primers 12S-FW (5'-GGTAAATCTCGTGCCAGCCA-3') and 12S-REV (5'-TCCAGTATGCTTACCTTGTTACGAC-3') were designed for the amplification of a 12S rRNA conserved gene fragment, based on sequences available in the Genbank/EMBL database for various animals such as pig, cow, sheep and goat (Accession numbers AJ002189, J01394, NC_001941, and M55541, respectively). This set of primers was expected to produce amplicons of the same length (approximately 720 bp) in the 3 species analyzed in this work.

For PCR amplification, each reaction mixture (50 μL) contained 100 ng of template DNA, 2 mM MgCl_2 , 5 pmol of each primer, 200 μM of each dNTP, and 2U of Tth DNA polymerase (Biotools, Madrid, Spain) in a reaction buffer containing 75 mM Tris-HCl, (pH 9.0), 2 mM MgCl_2 , 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 35 cycles consisting of 30 s at 93°C for denaturation, 30 s at 63°C for primer annealing, and 45 s at 72°C for extension. The last extension step was 5 min longer.

PCR products (10 μL) were mixed with 2 μL gel loading solution (Sigma-Aldrich) and electrophoresed in a 1.5% D1 (Hispanlab S.A., Torrejón, Spain) agarose gel, containing 1 $\mu\text{g/mL}$ ethidium bromide in Tris-acetate buffer (0.04 M Tris-acetate and 0.001 M EDTA; pH 8.0) for 45 min at 100 V. The resulting DNA fragments were visualized by UV transillumination and analyzed using Geldoc 1000 UV Fluorescent Gel Documentation System-PC (Bio-Rad Laboratories, Hercules, CA).

Purification and Sequencing of PCR Products

Polymerase chain reaction products (90 μL) of the expected length obtained from cows', sheep's and goats' milk using oligonucleotides 12S-FW and 12S-REV were purified with the Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The DNA was eluted in 30 μL of sterile distilled water. The concentration of the PCR product was estimated by agarose gel electrophoresis using a standard (Mass Ruler; Bio-Rad) as reference marker. A Geldoc 1000 System-PC (Bio-Rad) was used for that purpose.

Sequences were determined at the DNA Sequencing Center (Facultad de Farmacia, Universidad Complutense, Madrid, Spain). The DNA sequencing was accomplished using dRhodamine terminator cycle sequencing ready reaction kit (Perkin-Elmer/Applied Biosystems Division, Foster City, CA) in an ABI Prism model 377 DNA sequencer (Perkin-Elmer).

Sequence analysis and alignments were performed using the Wisconsin Package, version 9.0 (Genetics Computer Group, Madison, WI).

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

Information obtained after alignment of cow, sheep, and goat 12S rRNA gene sequences was used to design the following primer pair, potentially suitable for the amplification of a cow-specific 223-bp DNA fragment:

12SM-FW

(5'-CTAGAGGAGCCTGTTCTATAATCGATAA-3'),

common to the 3 species analyzed in this work, and

12SBT-RV

(5'-TGGTTTCATAATAACTTTCGCGCT-3'),

specific for cow.

The specificity of the primer set was first checked by Genebank/EMBL database FASTA program and was confirmed by challenging it with milk samples of different breeds of the 3 considered species: cow ("Frisona", "Pardo Alpina", "Rubia Gallega", "Asturiana", and "Avisleña"), sheep ("Merina", "Manchega", "Lacha", "Churra", "Castellana", and "Carranzana"), and goat ("Murciana", "Majorera", and "Palmera") in preliminary PCR experiments. Once assessed the specificity of the primers and raw and heat-treated milk binary mixtures (cow in sheep and cow in goat) were tested for DNA amplification.

Double-stranded amplifications were carried out in a final volume of 50 μ L containing 100 ng of template DNA, 2 mM MgCl₂, 10 pmol of each primer, 200 μ M of each dNTP, and 2U of Tth DNA polymerase (Biotools) in a reaction buffer containing 75 mM Tris-HCL (pH 9.0), 2 mM MgCl₂, 50 mM KCL, 20 mM (NH₄)₂ SO₄, and 0.001% BSA. Amplification was carried out in a Progene Thermal Cycler (Technique Ltd.) programmed to perform a denaturation step of 93°C for 2 min, followed by 40 amplification cycles that 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.

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

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 cows' milk ranging from 0.1 to 100% (vol/vol).

RESULTS

In this work, the mitochondrial 12S rRNA gene was used as a target to develop a PCR-based method for the specific detection of cows' milk in sheep's and goats' milk. To amplify a DNA fragment from the 12S rRNA gene of the 3 species considered in this study (cow, sheep, and goat), primers *12S-FW* and *12S-REV* were designed based on sequences available in the Genbank/EMBL database for several animal species. As shown in Figure 1, PCR products of the expected length, approximately 720 bp, were obtained from cows', sheep's, and goats' milk samples when using oligonucleotides *12S-FW* and *12S-REV* under the PCR conditions described.

The information obtained after sequencing and alignment of the 12S rRNA fragments from cow, sheep, and goat allowed the design of a reverse primer specific for cow (*12SBT-RV*), complementary to a gene sequence in which differences with the other species were re-

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

markable. This primer, along with the conserved forward *12SM-FW* oligonucleotide, was expected to yield a cow-specific amplicon of 223 bp in the 12S rRNA gene.

As can be seen in Figure 2, DNA extracted from cows' milk was successfully amplified with *12SM-FW* / *12SBT-RV* primer pair, giving rise to the expected PCR fragment, whereas no amplification products were obtained with DNA from sheep and goat milk samples. For each species, DNA from different breeds was analyzed, confirming the usefulness of the selected primer pair for the specific identification of cows' target sequence.

The sensitivity of the assay for identification of cows' milk in sheep's and goats' milk was also evaluated as a part of this study. To determine the detection limit of the PCR, amplification reactions were performed on DNA extracted from 2 series of binary raw milk mixtures (cow in sheep and cow in goat) comprising 0.1, 0.5, 1, 5, 10, and 100% (vol/vol) of cows' milk. Figures 3 and 4 show the cow 223-bp bands obtained from cow-sheep and cow-goat milk mixtures, respectively, along with the relationship between template DNA amounts and band intensity. The detection threshold was 0.1% for both series of milk mixtures.

Milk mixtures of cow-sheep and cow-goat were also subjected to heat treatments of pasteurization (65°C,

Figure 3. Electrophoretic analysis of the 12S rRNA PCR products obtained from raw milk binary mixtures of cow in sheep using primers *12SM-FW* and *12SBT-REV*. Samples are cow 100% (lane 1), cow 10% (lane 2), cow 5% (lane 3), cow 1% (lane 4), cow 0.5% (lane 5), cow 0.1% (lane 6), and sheep 100% (lane 7). NC = negative control; M = molecular weight marker, 1 kb plus DNA ladder (GibcoBRL).

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 5 and 6).

DISCUSSION

The DNA-based methods applied to species identification in animal products have received particular attention in recent years. Methods currently used for milk species identification include the development of conserved mitochondrial or nuclear DNA primers for PCR amplification, followed by complementary techniques such as sequencing or PCR-RFLP (Branciari et al., 2000). Alternatively, specific primers have been successfully applied for the direct detection of target species in simplex or multiplex PCR formats (Rea et al., 2001; Bottero et al., 2002, 2003). In this work, a single-step PCR assay has been developed for the specific detection of cheaper bovine milk in ovine and caprine milk.

Many PCR-based assays use DNA targets in the mitochondrial genome. These non-nuclear targets possess

Figure 4. Electrophoretic analysis of the 12S rRNA PCR products obtained from raw milk binary mixtures of cow in goat using primers *12SM-FW* and *12SBT-REV*. Samples are cow 100% (lane 1), cow 10% (lane 2), cow 5% (lane 3), cow 1% (lane 4), cow 0.5% (lane 5), cow 0.1% (lane 6), and goat 100% (lane 7). NC = negative control; M = molecular weight marker, 1 kb plus DNA ladder (GibcoBRL).

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

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

several advantages over nuclear genes (Unsel et al., 1995): They are generally more abundant in any given sample than single-copy nuclear genes, and, because mitochondrial DNA has a relatively high mutation rate compared with nuclear DNA, they contain a greater accumulation of point mutations that can be used to better define species differences. Moreover, mitochondrial DNA tends to be inherited through the maternal germline, and the resulting lack of heterozygosity in the alleles under study simplifies analysis (Kocher et al., 1989). Therefore, the mitochondrial encoded gene for 12S rRNA was selected in this work as the target sequence for species identification.

Sequence comparison of 12S rRNA gene available in the GenBank/EMBL database for several animal species permitted the design of 2 conserved primers, *12S-FW* and *12S-REV*, which amplified a DNA fragment of approximately 720 bp from cows', sheep's, and goats' milk (Figure 1). The identity of the amplicons was confirmed after purifying and sequencing the PCR fragments obtained from 2 individuals of each considered species. Cow, goat, and sheep sequences were 100% identical to their homologous obtained from the GenBank/EMBL (data not shown).

Species-specific PCR has shown to be a suitable method to control food authenticity because a specific target sequence can be detected even in matrices containing a pool of heterogeneous genomic DNAs, such as milk mixtures or other dairy products (Tartaglia et al., 1998). However, the extraction of inhibitor-free DNA from milk samples, capable of being amplified optimally by PCR, has been reported as a common problem (Lipkin et al., 1993; Amills et al., 1997; Murphy

et al., 2002). In the present work, an approach was developed for removing PCR inhibition factors from milk, consisting of a "milk clearing solution" previously used in our laboratory for the collection of bacteria from raw milk samples (Gutiérrez et al., 1997). The milk clearing reagent also facilitated the recovery of somatic cells from milk without components that inhibit the PCR. This procedure, followed by a DNA extraction protocol based on a commercial kit, proved to be highly efficient, yielding good quality DNA even when milk samples were submitted to severe heat treatments.

To make the detection unequivocal, a reverse primer specific for cow (*12SBT-REV*) was designed following sequence alignment and comparison. As expected from sequence analysis, the *12SBT-REV* primer, together with the forward *12SM-FW* conserved oligonucleotide, amplified a 223-bp fragment from cows' DNA, whereas no amplification bands were detected in sheep and goat species (Figure 2).

Once the specificity of the primer pair was confirmed, PCR amplification was performed on binary raw milk mixtures (cow in sheep and cow in goat) comprising 0.1, 0.5, 1, 5, 10, and 100% (vol/vol) of cows' milk to determine the detection limit of the assay in milk samples. It was observed that the lower the percentage of the target species (cow) in the mixture, the fainter the band obtained in the PCR with the cow-specific primer pair. The detection limit (lower milk percentage yielding visible DNA amplification) of the assay was 0.1% of cows' milk in both cow-sheep (Figure 3) and cow-goat (Figure 4) milk mixtures.

Finally, the effect of thermal treatment of milk on the technique's ability to detect species was studied 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 cows' milk in the PCR developed were not substantially modified with respect to raw milk mixtures (Figures 5 and 6).

In conclusion, the PCR method described herein is a useful and straightforward approach for the detection of low levels of cows' milk substitution (0.1%) in milks of a higher commercial value. Moreover, the technique offers high sensitivity and specificity, with the great advantage that it can also be applied in thermally or otherwise processed milk products with extensive protein denaturation. Therefore, this PCR technique may prove useful for food inspection services to trace the species origin of milk mixtures or cheese products, thereby protecting the traditional manufacturers and consumers against food product adulteration and misrepresentation.

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REFERENCES

- Addeo, F., L. Moio, L. Chianese, C. Stingo, P. Resmini, I. Berner, I. Krause, A. Di Luccia, and A. Bocca. 1990. Use of plasmin to increase the sensitivity of the detection of bovine milk in ovine and/or caprine cheese by gel isoelectric focusing of γ_2 -caseins. *Milchwissenschaft* 45:708–711.
- Amills, M., O. Francino, M. Jansa, and A. Sánchez. 1997. Isolation of genomic DNA from milk samples by using Chelex resin. *J. Dairy Res.* 64:231–238.
- Anguita, G., R. Martín, T. García, P. Morales, A. I. Haza, I. González, B. Sanz, and P. E. Hernández. 1996. Immunostick ELISA for detection of cow's milk in ewe's milk and cheese using a monoclonal antibody against bovine β -casein. *J. Food Prot.* 59:436–437.
- Bottero, M. T., T. Civera, A. Anastasio, R. M. Turi, and S. Rosati. 2002. Identification of cow's milk in "buffalo" cheese by duplex polymerase chain reaction. *J. Food Prot.* 65:362–366.
- Bottero, M. T., T. Civera, D. Nucera, S. Rosati, P. Sacchi, and R. M. Turi. 2003. A multiplex polymerase chain reaction for the identification of cows', goats' and sheep's milk in dairy products. *Int. Dairy J.* 13:277–282.
- Branciarri, R., I. J. Nijman, M. E. Plas, E. Di Antonio, and J. A. Lenstra. 2000. Species origin of milk in Italian Mozzarella and Greek Feta cheese. *J. Food Prot.* 63:408–411.
- Calvo, J. H., R. Osta, and P. Zaragoza. 2002. Species-specific amplification for detection of bovine, ovine and caprine cheese. *Milchwissenschaft* 57:444–446.
- Chianese, L., P. Laezza, L. A. Smaldone, C. Stingo, L. Del Giovine, and F. Addeo. 1990. Evaluation of bovine milk in the buffalo Mozzarella cheese by two-dimensional electrophoresis. *Sci. Tec. Latt. Casear.* 41:315–326.
- Chikuni, K., T. Tabata, M. Kosugiyama, M. Monma, and M. Saito. 1994. Polymerase chain reaction assay for detection of sheep and goat meats. *Meat Sci.* 37:337–345.
- De Noni, I., A. Tirelli, and F. Masotti. 1996. Detection of cows' milk in non-bovine cheese by HPLC of whey protein: Application to goat milk cheese. *Sci. Tec. Latt. Casear.* 47:7–17.
- EC Regulation No. 1081/96 of the Commission June 14 (1996). Reference method for detection of cow's milk and cow's milk casein in cheese made from ewes, goats and buffalo milk or mixtures of ewes, goats and buffalo milk. *Official Journal of the European Commission*. L142, pp. 15–25.
- Gutiérrez, R., T. García, I. González, B. Sanz, P. E. Hernández, and R. Martín. 1997. A quantitative PCR-ELISA for the rapid enumeration of bacteria in refrigerated raw milk. *J. Appl. Microbiol.* 83:518–523.
- Haza, A. I., P. Morales, R. Martín, T. García, G. Aguita, I. González, B. Sanz, and P. E. Hernández. 1997. Use of a monoclonal antibody and two enzyme-linked immunosorbent assay formats for detection and quantification of the substitution of caprine milk for ovine milk. *J. Food Prot.* 60:973–977.
- Kocher, T. D., W. K. Thomas, A. Mayer, S. V. Edwards, S. Pääbo, F. X. Villablanca, and A. C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA* 86:6196–6200.
- Koh, M. C., C. H. Lim, S. B. Chua, S. T. Chew, and S. T. W. Phang. 1998. Random amplified polymorphic DNA (RAPD) fingerprints for identification of red meat animal species. *Meat Sci.* 48:275–285.
- Lipkin, E., A. Shalom, H. Khatib, M. Soller, and A. Friedmann. 1993. Milk as a source of deoxyribonucleic acid and as a substrate for the polymerase chain reaction. *J. Dairy Sci.* 76:2025–2032.

- Matsunaga, T., K. Chikuni, R. Tanabe, S. Muroya, K. Shibata, J. Yamada, and Y. Shinmura. 1999. A quick and simple method for the identification of meat species and meat products by PCR assay. *Meat Sci.* 51:143–148.
- Maudet, C., and P. Taberlet. 2001. Detection of cows' milk in goats' cheeses inferred from mitochondrial DNA polymorphism. *J. Dairy Res.* 68:229–235.
- Moio, L., M. L. Sasso, L. Chianese, and F. Addeo. 1990. Rapid detection of bovine milk in ovine, caprine and water buffalo milk or cheese by gel isoelectric focusing on phastsystem. *Ital. J. Food Sci.* 3:185–190.
- Molina, E., P. J. Martín-Álvarez, and M. Ramos. 1999. Analysis of cows', ewes' and goats' milk mixtures by capillary electrophoresis: Quantification by multivariate regression analysis. *Int. Dairy J.* 9:99–105.
- Murphy, A. M., M. Reza Shariflou, and C. Moran. 2002. High quality genomic DNA extraction from large milk samples. *J. Dairy Res.* 69:645–649.
- Rea, S., K. Chikuni, R. Branciari, R. Sukasi Sangamayya, D. Ranucci, and P. Avellini. 2001. Use of duplex polymerase chain reaction (duplex PCR) technique to identify bovine and water buffalo milk used in making Mozzarella cheese. *J. Dairy Res.* 68:689–698.
- Rodríguez, M. A., T. García, I. González, L. Asensio, B. Mayoral, I. López-Calleja, P. E. Hernández, and R. Martín. 2003. Identification of goose, mule duck, chicken, turkey, and swine in foie gras by species-specific polymerase chain reaction. *J. Agric. Food Chem.* 51:1524–1529.
- Tartaglia, M., E. Saulle, S. Pestalozza, L. Morelli, G. Antonucci, and P. A. Battaglia. 1998. Detection of bovine mitochondrial DNA in ruminant feeds: A molecular approach to test for the presence of bovine-derived materials. *J. Food Prot.* 61:513–518.
- Unsel, M., D. Beyermann, P. Brandt, and R. Hiesel. 1995. Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. *PCR Methods Appl.* 4:241–243.