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Rapid and Sensitive Identification of Buffalo's, Cattle's and Sheep's Milk using Species-Specific PCR and PCR-RFLP Techniques

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Abstract

For the rapid, specific and sensitive identification of buffalo's, cattle's and sheep's milk, species-specific PCR and PCR-RFLP techniques were developed. DNA from small amount of fresh milk (100 µl) was extracted to amplify the gene encoding species-specific repeat (SSR) region and the mitochondrial DNA segment (cytochrome *b* gene). PCR amplification size of the gene encoding SSR region was 603 bp in both buffalo's and cattle's milk, while in sheep's milk was 374 bp. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used to discriminate between buffalo's and cattle's milk. Restriction analysis of PCR-RFLP of the mitochondrial cytochrome *b* segment (359 bp) analysis showed difference between buffalo's and cattle's milk. Where, the fragment length (bp) generated by *Taq*I PCR-RFLP were 191 and 169, whereas no fragments were obtained in cattle's milk for cytochrome *b* gene (359 bp). The proposed PCR and PCR-RFLP assays represent a rapid and sensitive method applicable to the detection and authentication of milk species-specific.

Key words: milk, identification, species-specific, SSR, cytochrome *b*, PCR, PCR-RFLP

Introduction

Species identification of milk products have received considerable attention over the last five 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). The 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. To avoid unfair competition and to assure consumers of accurate labeling, it is necessary to develop techniques for assessing if the species or the percentage of milk in a milk mixture corresponds to the legal requirement (Calvo et al., 2002). Many different analytical approaches, such as immunological, electrophoretic, and chromatographic techniques, have been developed for species identification of milk and dairy products (Addeo et al.,1990, Chianese et al.,1990, Moio et al.,1990 and Molina et al.,1999). However, electrophoretic and immunological methods are often not suitable for food products with complex matrices, being also significantly less sensitive in heat-treated material.

Because of milk contains large number of somatic cells (leukocytes and epithelial mammary cells) from mammary glands, recent studies have shown that it is possible to isolate DNA

from these cells, which can be successfully applied for fast and sensitive species differentiation using molecular genetic techniques (Lipkin et al., 1993; Amills et al., 1997; Maudet and Taberlet, 2001). In the present study, rapid and sensitive species-specific PCR and PCR-RFLP techniques were developed for identification and detection of buffalo's, cattle's and sheep's milk.

Materials and methods

Buffalo's, cattle's and sheep's genomic DNA included mitochondrial DNA (mt-DNA) was extracted from very small fresh milk samples according to Sharma et al. (2000) with some modifications. However, 1400 µl of lyses buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0 and 0.5% SDS) and 30 µl of proteinase K (20 mg/ml) were added to 100 µl of each milk sample. The mixture was vortexed and incubated at 37°C overnight. DNA was extracted by equal volumes of phenol-chloroform- isoamylalcohol (25:24:1) and chloroform-isoamylalcohol (24:1), successively. DNA was precipitated by adding two equal volumes of chilled ethanol (95%) in the presence of a high concentration of salts (10% 3 M sodium acetate). The pellet was washed with 70% ethanol, air-dried and subsequently dissolved in an appropriate volume (100 µl, approximately) of double distillated water (ddH_2O).

SSR gene (603 bp) and the segment of mt-DNA (359 bp) were amplified with the use of primers sequences as can be seen in Table 1 (Lenstra et al., 2001 and Abdel-Rahman, 2006). PCR was performed in a reaction volume of 25 µl contained 50 ng of genomic DNA, 25 pmol dNTPs, 25 pmol of the primer, 1.0 U *Taq* DNA polymerase and reaction buffer (Finnzymes). After 35 cycles (94°C, 30 sec; 60°C for SSR gene and 56°C for cytochrome *b* gene, 30 sec; 72°C, 30 sec), the presence of the 603 bp and 359 bp products were checked on 3% agarose gel. For restriction analysis, digestion of 10 µl of each PCR product (359 bases of mitochondrial cytochrome *b* gene) was accomplished with 10 units *Taq*I restriction enzyme for one hour at 65°C. Digested DNA was separated on 3% agarose gels in 1x TBE buffer, stained with ethidium bromide, visualized under UV light and photographed by Gel Documentation system (Alpha Imager M1220, Documentation and Analysis System, Canada).

Table 1: Primer sequences and annealing temperatures of buffalo's, cattle's and sheep's SSR and cytochrome *b*.

Specie	Primer sequence 5' → 3'	Annealing temperature
Buffalo's and cattle's SSR	AAGCTTGTGACAGATAGAACGAT CAAGCTGTCTAGAATTCAGGGA	60 °C
Sheep's SSR	GTTAGGTGTAATTAGCCTCGCGAGAA AAGCATGACATTGCTGCTAAGTTC	62 °C
Buffalo's, and cattle's cytochrome *b*	CCATCCAACATCTCAGCATGATGAAA GCCCCTCAGAATGATATTTGTCCTCA	57 °C

Results and discussion

In the present study, genomic DNA included mitochondrial DNA (mt-DNA) from milk of buffalo, cattle and sheep was extracted to amplify both the gene encoding species-specific

repeat (SSR) and the gene encoding cytochrome *b*. PCR amplification of the gene encoding species-specific repeat (SSR) yielded 603-bp in length in both buffalo and cattle, whereas in sheep was 374-bp (Figure 1). As can be seen, the size and the position of the PCR-SSR generated fragment (603-bp) with both buffalo and cattle are exactly the same with the fragment of the molecular weight marker (ΦX174 DNA ladder).

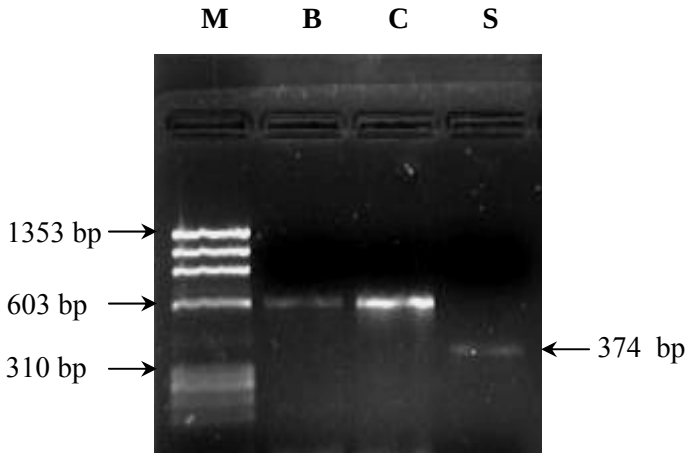

Figure 1: PCR products (603 and 374-bp) generated by primers species-specific oligonucleotide. Lane **B** is buffalo, lane **C** is cattle, lane **S** is sheep and lane **M** is a molecular weight marker (ΦX174 DNA – *Hae* III Digest).

For differentiation between buffalo's and cattle's milk, PCR-RFLP technique for cytochrome *b* gene was used. However, the amplification product of the gene encoding cytochrome *b* in both cattle and buffalo was 359-bp in length. Two different patterns were generated or yielded after the *Taq*I restriction enzyme digestion and the sizes were 191- and 169-bp only with buffalo's amplified cytochrome *b* gene, while with cattle was not digested (359-bp) allowing an identification of buffalo's and cattle's milk (Figure 2).

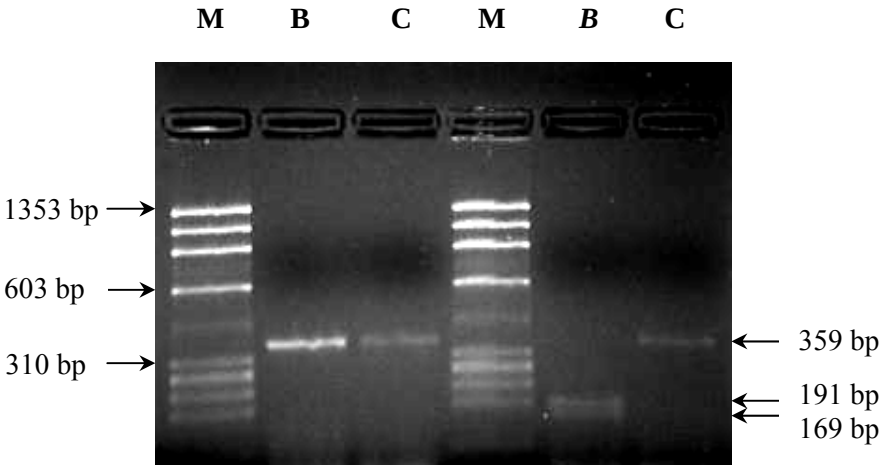

Figure 2: Agarose gel electrophoresis of amplified cytochrome *b* gene (lane **B**: buffalo and lane **C**: cattle) following digestion with *Taq*I generated two fragments with a size of 191 and 169 bp (lane ***B***: buffalo). Lane **M** is a molecular weight marker (ΦX174 DNA – *Hae* III Digest).

This research was mainly performed for milk identification of some farm animal species, such as buffalo, cattle and sheep using species-specific PCR and PCR-RFLP techniques. The results of these two techniques showed good evidence for molecular markers linked to genetic diversity among buffalo, cattle and sheep concerning kind of milk. Where, PCR amplification of the species-specific repeat (SSR) gene yielded two different PCR fragments (603 for buffalo and cattle and 374 bp for sheep). On the other hand, PCR amplification of the gene encoding cytochrome *b* gene generated the same fragment (359 bp) in both cattle and buffalo. To discriminate between buffalo's and cattle's milk, *Taq*I digestion of the PCR fragment (359 bp) resulted two restriction pattern fragments 191- and 169-bp in buffalo, but digested not in cattle. After having discussed species-specific PCR and PCR-RFLP techniques, the results provide us with a rapid, sensitive and straightforward approach applicable to the identification and the authentication of milk and other dairy products in routine analysis.

In a study to find molecular markers for Prim 'Holstein's milk detection in RDO (Registered Designation of Origin) cheese, four genes affecting coat color in cattle (c-kit, MGH, TYRP1 and MC1R) have been sequenced for three mountain breeds and the Prim 'Holstein breed (Maudet and Taberlet, 2002). The author could find that the Only MC1R gene (E-locus) has shown variation between the four breeds. As a consequence, DNA extraction from cheese, a preamplification of the gene and a competitive oligonuleotide priming PCR on MC1R mutations were performed. This approach provided the author a good reproducibility with a detection limit of about 1% of Holstein's milk in milk curd. For quantification of bovine milk in ovine cheeses, a duplex polymerase chain reaction method was used by Mafra et al., (2004). This method was based on the mitochondrial 12S and 16S rRNA genes to generate fragments of different lengths. Where, a linear normalized calibration curve was obtained between the log of the ratio of the bovine band intensity and the sum of bovine and ovine band intensities versus the log of cow's milk percentage. However, the proposed duplex PCR provided the author a simple, sensitive, and accurate approach to detect as low as 0.1% bovine milk in cheeses and to quantify bovine milk in ovine cheeses in the range of 1-50%.

Eventually, Lopez-Calleja et al., (2004) developed a polymerase chain reaction (PCR) assay for the specific identification of cow's milk in sheep's and goat's milk by using primers targeting the mitochondrial 12S rRNA gene. 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 goat's 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 cow's milk with a good sensitivity threshold (0.1%).

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