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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 168, whereas no fragments were obtained in cattle's milk for cytochrome-*b* gene (359 bp). The proposed PCR and PCR–RFLP assays rep resent a rapid and sensitive method applicable to the detection and authentication of milk species-specific.

Keywords: Milk; Identification; Species-specific; SSR; Cytochrome-*b*; PCR; PCR–RFLP

1. Introduction

Species identification of milk products have received considerable attention over the last five years. Particularly, government regulations (Bottero et al., 2003) and species identification of dairy products has a remarkable importance for several reasons, including frequent human adverse reactions toward some species milk proteins. 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, Osta, & Zaragoza, 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, Sasso, Chianese, & Addeo, 1990; Molina, Martín-A'lvarez, & Ramos, 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.

In essence, milk contains large number of somatic cells (leukocytes and epithelial mammary cells) from mammary glands. These somatic cells play a protective role against infectious disease in the dairy animals mammary gland (Marcus, Kehrli, & Shuster, 1994). On addition of somatic cells play a protective role against infectious disease, the somatic cell counts have legal, milk quality and productivity implications and each is important Therefore, an important point of emphasis should be that the European Union, New Zealand, Australia and a few other countries have

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adopted a standard for maximum allowable cell counts in their grade a type milk of 400 000 cell/ml (e.g., dairy cows). Canada presently is at 500 000 cell/ml while the US has an allowable legal maximum of 750 000 cell/ml (Ingalls, 1998).

Somatic cells in milk were used as a source of DNA which can be successfully applied for fast and sensitive species differentiation using molecular genetic techniques (Amills, Francino, Jansa, & Sa'nchez, 1997; Lipkin, Shalom, Khatib, Soller, & Friedmann, 1993; Maudet & 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.

2. 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, Appa Rao, and Totey, 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 and the segment of mt-DNA were amplified with the use of primers sequences as can be seen in Table 1 (Abdel-Rahman, 2006; Lenstra, Buntjer, & Janssen, 2001). 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 s; 60 °C for SSR gene and 56 °C for cytochrome-*b* gene, 30 s; 72 °C, 30 s), the presence of the 603 bp and 359 bp products were checked on 2% 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 2% agarose gels in IX TBE buffer, stained with ethidium bromide, visualized under UV light and photographed by Gel Documentation system (Alpha Imager Ml220, Documentation and Analysis System, Canada).

3. Results

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 (Fig. 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).

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 bp and 168 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 (Fig. 2).

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 bp 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 bp and 168 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

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

Species	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