PCR-based detection of cow and goat milk in sheep milk and dairy products marketed in Mashhad city of Iran

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Abstract:

BACKGROUND: The extensive consumption of milk and dairy products makes these foodstuffs targets for potential adulteration with financial gains for unscrupulous producers. **OBJECTIVES:** The aim of this study was using PCR assay to detect cow milk in labeled sheep milk, sheep yoghurt, and Lighvan cheese (a traditional ripened cheese produced from sheep's milk). **METHODS:** The assay utilized primers targeting the mitochondrial 12s and 16s rRNA gene. In this study, 35 samples of sheep milk, 35 samples of sheep yoghurt, and 35 samples of Lighvan cheese were purchased from different supermarkets in Mashhad city with different batch numbers. **RESULTS:** The results showed only 21 out of 105 (20%) samples contained pure sheep milk. Undeclared presence of cow and goat milk was detected in 33(31.5%) and 68(65%) of the 105 samples, respectively. **CONCLUSIONS:** It seems the PCR based analytical method is an applicable technique to monitor adulteration in dairy products.

Introduction

The "Farm to Fork" concept implies the traceability and authenticity of a product from raw material to consumption. To guaranty the food authenticity, the development of analytical techniques to enable authorities and producers to check if the products are correctly described and labeled is necessary (Santos et al., 2003).

In dairy products market, the substitution of primary ingredients, typically cow milk for sheep milk or goat milk, may be a temptation when there is seasonal oscillations and much lower ovine milk yield and also the much lower price of bovine milk (Mafra et al., 2004). Although Cows milk dominates the market, and for the majority of people it is the only milk that is ingested. Reasons for which cows milk is avoided include: intolerance or allergy (Halken, 2003; Sampson, 2003); religious, ethical or cultural objections (Shatenstein and Ghadirian, 1998); personal preference; and unsuitability for special products (Hurley et al., 2004).

Different analytical approaches have been applied for milk species identification among which immunological (Xue et al., 2010; Zelenakova et al., 2008; Hurley et al., 2004), electrophoretical (Mayer, 2005), chromatographic (Enne et al., 2005) are worth mentioning. Ferreira and Caçote (2003) reported that the RP-HPLC is a very sensitive and accurate method for studying milk percentage, as well as fresh and ripened cheeses made from binary mixtures of cow, sheep, or goat raw milk. Urbanke et al. (1992) have also used RP-HPLC to control milk adulteration. Recently, attention has been turning towards DNA-based methods for many aspects of food authentication, including milk adulteration detection (Plath et al., 1997; Lockley and Bardsley, 2000; Woolfe and Primrose, 2004). Polymerase Chain Reaction (PCR) is one of the most used molecular biology tools and

has been used by many authors for species identification in raw meat, meat products, fish, and dairy products (Herrero-Martínez et al., 2000; Branciari et al., 2000; Bania et al., 2001; Maudet and Taberlet, 2001; Veloso et al., 2002). Genomic DNA in somatic cells persists in milk and even ripened cheese. PCR of DNA extracted from somatic cells in milk has been successfully applied to detect adulteration of milk products, targeting DNA sequences with adequate species-species variation (Plath et al., 1997; Bania et al., 2001; Klotz and Einspanier, 2001; Maudet and Taberlet, 2001; Rea et al., 2001; Bottero et al., 2002, 2003). Many of these studies utilize mitochondrially-encoded genes such as the cytochrome b gene, because the sequences have been shown to differ by a number of nucleotides between even closely related species (Herman, 2001; Bottero et al., 2003).

The aim of the present study was to differentiate the milk of three closely related species (goat, sheep and cow) sold as sheep milk, sheep yoghurt, and Lighvan cheese (a traditional ripened cheese produced from sheep milk) in Mashhad city dairy markets, using multiplex PCR assay.

Materials and Methods

Sampling: Considering that 5% detection limit is sufficient for the proof of undeclared milk component, and adulteration of less than 5% lacks any economic effect (Moskova and Paulickova, 2006; Cozzolino et al., 2001), we considered this detection limit as sufficient in our study.

In order to evaluate the applicability of the test, and as positive control, ten series of milk mixture were prepared in our laboratory under controlled conditions, using the following types: pure cow, pure sheep and pure goat milk and a mixture of 5% of goat milk in sheep milk, 5% of goat milk in cow milk, 5% of sheep milk in cow milk, 5% of sheep milk in goat milk, 5% of cow milk in sheep milk, 5% of cow milk in goat milk and a mixture of cows/sheep/goat milk with the same portions. 35 Lighvan cheese samples, 35 samples of sheep yoghurt, and 35 samples of sheep milk which were labeled "prepared from pure sheep milk" were purchased from different supermarkets in Mashhad city with different batch numbers.

DNA extraction: DNA from milk mixtures, yoghurts, and cheese samples was extracted using the First- Magnetic Milk Kit (Gen-ial, Germany) protocol as indicated by the manufacturer. The DNA was quantified by spectrophotometry (Ultraspec 2000 Pharmacia Biotech) and diluted to 50 ng mL-1.

Primers: Specific primers for caprine, ovine, and bovine species, which targeted the 12s and 16s mitochondrial rRNA and were designed by Buttero et al. (2003) were used in this study. These species-specific primers (synthesized by Bioneer, South Korea) are capable to generate species-specific amplicons with different lengths in which differences between the caprine, ovine, and bovine's milk product's origin were distinguishable (Table 1).

Multiplex PCR: In order to simultaneously detect each animal species, all primer sets were used to develop a one-step reaction. Amplifications were carried out in a final volume of 50 µL containing 10mM Tris-HCl (pH 8.3), 2 unit AmpliTaq Gold DNA polymerase (PE Applied Biosystems), 0.2mM each of dATP, dCTP, dGTP, dTTP (Pharmacia), 2.5mM MgCl2, 25, 30, 15 pmol of primers, respectively, of bovine, caprine and ovine origin (Table 1), and 250 ng of DNA template. After an initial denaturation step at 94°C for 5 min, 35 cycles were programmed as follows: 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and the final extension at 72°C for 5 min. Amplimers were resolved on 2.5% agarose electrophoresis, carried out in Tris acetate EDTA buffer for 60 min at 120V and stained with ethidium bromide (0.4 mg mL-1 for 20 min).

Results

The specific fragments of 256 bp, 326 bp and 172 bp were amplified for bovine, caprine, and ovine materials respectively (Figure 1). The results of m-PCR analyses of 35 samples of each labeled sheep milk, sheep yoghurt, and Lighvan cheese are demonstarted in Table 2. Only 21 out of 105 (20%) samples contained pure sheep milk. Undeclared presence of cow and goat milk was detected in 33 (31.5%) and 68(65%) of the 105 samples, respectively.

Discussion

There is a growing demand from consumers for authentic and correctly labeled milk and cheese, particularly for genuine traditional products (Moatsou

Table 1. Oligonucleotides used as m-PCR primers (Bottero et al., 2003).

Species and genes	Oligonucleotide primers	Amplicons
Ovis aries (12s gene for sense and 16s gene for antisense) NC001941a	(F) 959 (50 ATATCAACCACACGAGAGGAGAC 30) (R) 1130 (50 TAAACTGGAGAGTGGGAGAT 30)	172 bp
Capra hircus (12s gene for sense and antisense) M55541a	(F) 144 (50 CGCCCTCCAAATCAATAAG 30) (R) 469 (50 AGTGTATCAGCTGCAGTAGGGTT 30)	326 bp
Bos taurus (12s gene for sense and antisense) NC001567a	(F) 916 (50 GTACTACTAGCAACAGCTTA 30) (R) 1171 (50 GCTTGATTCTCTTGGTGTAGAG 30)	256 bp

Table 2. The results of m-PCR from labeled sheep milk, sheep yoghurt and Lighvan cheese samples using species-specific primers.

Samples	Samples containing sheep milk		Samples containing sheep and goat milk		Samples containing sheep and cow milk		Samples containing sheep, goat and cow milk		Total
	N	%	N	%	N	%	N	%	
Sheep milk	6	17	16	45.7	3	8.6	10	28.6	35
Lighvan cheese	11	31.4	14	40	8	22.9	2	5.7	35
Sheep yoghurt	4	11.4	21	60	5	14.3	5	14.3	35
Total	21		51		16		17		105

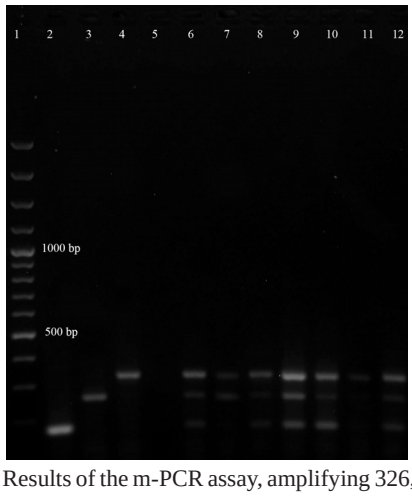

Figure 1. Results of the m-PCR assay, amplifying 326, 256, and 172 base pairs caprine, bovine, and ovine material, respectively. Lane1: 100bp marker, Lanes 2, 3, and 4: positive control, Lane 5: negative control, Lanes 6-12 dairy products samples.

and Anifantakis, 2003). Therefore, it is important to protect the consumer by ensuring that adequate control measures are in place, and that the food analyst has suitable methods for the detection of milk adulteration. Labeling and authenticity regulations may differ from country to country, which entail the need for analytical tests to enforce such policies (Dennis, 1998). Although there is no report of adulteration in milk and dairy products from Iran, there are several reports from other countries; for example, Di-Pinto et al. (2004) analyzed 30 mozzarella cheeses and the presence of cow milk was found in 22 samples. The presence of cow milk was detected in 67.3% of sheep and goat cheeses in Romania by Stanciuc and Rapeanu (2010), and cow milk was detected in 48 % of cheese samples by Colak et al. (2006). According to Zelenakova et al. (2009), from 20 sheep milk samples, cow milk occurrence was detected in 8 samples and from 30 samples of sheep cheese, 12 samples contained a mixture of cow milk.

Different methods based on protein analysis are currently used for milk species identification. Although these techniques are of considerable value in certain instances, the success of analytical tools that rely on protein detection for species identification may be in some cases hindered by proteolysis or denaturation of milk proteins as a result of heat treatment and cheese maturation (Plath et al., 1997). Another disadvantage of these techniques is that they are time consuming and laborious and the increased requirements for sample handling during preparation can adversely affect the quality of the analysis (Karoui and Baerdemaeker, 2007).

Recently, full attention has been turning towards application of DNA-based approaches for the authentication of food. Particularly, the polymerase chain reaction (PCR) is becoming increasingly used for the specific detection of the animal origin in milk and cheese products. Somatic milk cells, principally represented by leucocytes, still persist during cheese manufacturing processes and can be used as a source of amplifiable DNA (Diaz et al., 2007).

Among the target gens, the mitochondrial gene coding cytochrome b, which is specific for mammals, and 12S rRNA and 16S rRNA have been widely used (Maskova and Paulickova, 2006; Mafra et al., 2007; Bottero et al., 2003).

In order to determine the animal species in dairy

products, using molecular methods, one strategy is to use universal primers and subsequently RFLP the amplicon. When complex mixtures of two or more species are to be detected, the interpretation of RFLP results could be difficult due to overlapping restriction patterns, which may be generated. Moreover, efficiency of restriction digestion must be carefully checked since a small portion of undigested amplicon might be regarded as a contaminating species lacking the restriction site. By multiplex PCR, which has been used in the present study, complex mixtures can be detected in a single step, provided that specific amplicons are of different length and easily resolved by agarose gel electrophoresis. With regard to dairy products, multiplex PCR can be advantageously applied, as only a few species are generally involved (cow, goat, sheep and buffalo), and it is hardly likely that other unknown species would be present. Primers design strategy was addressed to two different genes (12s rRNA and 16s rRNA) of mitochondrial DNA, characterized by alternate well-conserved regions and also variable regions. Primer binding sites were selected for each species in order to generate specific amplimers of different lengths (Bottero et al., 2003). Although the primers have been designed for European breeds, our study proved that these primers could recognize Iranian breeds as well. As nonauthentic milk products are produced potentially for financial gain (Maudet and Taberlet, 2001), adulterating either goat or sheep milk with cow milk for less than 5% does not sound to be economical. In our experiment the assay detected 5% of caw milk in sheep or goat milk, although it has been claimed that minimum detection level of used multiplex PCR is 0.5% (Bottero et al., 2003). The possibility of detecting lower levels of contaminating milk in dairy products would be interesting from a theoretical point of view, but not helpful in practice. In fact, in the case of very small amounts of contaminating milk, it could be difficult to establish exactly whether a fraud is presumable or, rather, an unintentional contamination might be supposed. However, attempts to use PCR as a quantitative tool for food authentication are still very scarce (Jooyandeh and Aberoumand, 2010) because in the case of mastitis or subclinical mastitis the somatic cells which harbor the target gen increases significantly.

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تشخیص تقلب در شیر و محصولات لبنی ایران با استفاده از روش PCR

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چکیده

زمینه مطالعه: مصرف گسترده شیر و فراورده های لبنی، این محصولات را به هدفی برای تقلب وسودجویی توسط تولیدکنندگان غیر متعهد تبدیل کرده است. **هدف:** هدف از این مطالعه ارزیابی روش PCR جهت تشخیص حضور شیر گاو در شیر، ماست و پنیر لیقوان گوسفندی انجام گردید. **روش کار:** در این بررسی از پرایمرهایی که ژن های 12s و 16s RNA میتوکندریایی را مورد هدف قرار می دادند استفاده شد. در این مطالعه ۳۵ نمونه شیر گوسفند، ۳۵ نمونه ماست گوسفند و ۳۵ نمونه پنیر لیقوان گوسفندی از بهرهای تولید متفاوت از سوپر مارکت های مختلف در سطح شهر مشهد خریداری گردید. **نتایج:** نتایج نشان دادند که تنها ۲۱ نمونه از ۱۰۵ نمونه (۲۰٪) مورد آزمایش از شیر خالص گوسفندی تهیه شده بود. حضور شیر گاو و بز به ترتیب در ۳۳ (۳۱/۵٪) و ۶۸ (۶۵٪) نمونه از نمونه های مورد بررسی تشخیص داده شد. **نتیجه‌گیری نهایی:** به نظر می رسد که روش تشخیص بر اساس PCR متدی مناسب برای پایش تقلب در محصولات لبنی باشد.

واژه‌های کلیدی: تقلب، پنیر لیقوان، شیر، واکنش زنجیره ای پلیمراز، ماست

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