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Fraud Identification of Undeclared Milk Species in Composition of Sheep Yogurt and Cheese Using Multiplex PCR Assay

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Abstract

Background: One of the adulterant practice in dairy industry is the use of a less costly type of milk such as cow and goat milk instead of more expensive ones especially sheep milk. The aim of the present study was to assess fraud identification of undeclared milk species in composition of sheep yogurt and cheese offered as "pure sheep" in Iranian local markets.

Methods: Samples of sheep yogurt (n=40) and cheese (n=40) were purchased randomly from supermarkets in Ahvaz, Iran. According to the information provided by the vendors, all samples contained pure sheep milk. Using species-specific primers, a multiplex PCR assay was performed to detect the fraudulent addition of cow's or goat's milk into the sheep products.

Results: In the molecular assay, the limit of detection of cow's or goat's milk in sheep yogurt was 2%, while this limit was 4% in sheep cheese. Only 27.5% of the yogurt samples and 20% of the cheese samples contained pure sheep milk. Adulterant presences of cow's and goat's milk were detected in 37.5% and 22.5% of the yogurt and 35% and 17.5% of the cheese samples, respectively.

Conclusion: We showed high level of adulteration in the retail trade of sheep dairy products in Ahvaz, Iran. Therefore, continual surveillance on the production and sale of these products should be considered. Also, it was found that the multiplex PCR method used in this study is a useful and straightforward approach for the detection of low levels of cow's or goat's milk in sheep products.

Introduction

Illegal adulteration of raw materials to be used for the commercial preparation of food is a common problem. The "Farm to Fork" concept implies the traceability and authenticity of a product from raw material to consumption. In the dairy industry, a widespread adulterant practice is the use of a less costly type of milk instead of more expensive ones. An eminent example is the addition of cow's milk into the sheep's, goat's or buffalo's milk or other dairy products that are faultily labeled "pure sheep," "pure goat," or "pure buffalo" (Bottero et al., 2003; Calvo et al., 2002; Di Pinto et al., 2004; Lopez-Calleja et al., 2004). Development of analytical techniques to enable authorities and producers to check if the products are correctly described and labeled is required (Hurley et al., 2004; Xue et al., 2010; Zelenakova et al., 2009). Among many different analytical approaches

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which have been used for species identification of milk and dairy products, PCR-based methods are the most reliable and sensitive techniques. PCR-based methods currently used for milk species identification include the development of specific primers for conserved nuclear or mitochondrial DNA in order to PCR amplification, followed by some supplementary techniques such as sequencing or RFLP. Alternatively, specific primers have been successfully applied for the direct detection of target species in simplex or multiplex PCR formats (Abdel-Rahman and Ahmed, 2007; Bottero et al., 2002; Branciari et al., 2000; Lopez-Calleja et al., 2004; Mafra et al., 2004; Rea et al., 2001).

Nowadays, the majority of retail yogurts and cheeses are made from cow's milk. Pure sheep yogurt and cheese is popular because of their special taste and flavor. However, the supply of these products fluctuates with the year season due to the reproductive cycle of the animals. The increased demand for these products as well as the higher prices, have made the substitution or admixture of sheep's milk with goat's or cow's milk. Therefore, the aim of the present study was to assess fraud identification of undeclared milk species in composition of sheep yogurt and cheese offered as "pure sheep" in the local markets, using multiplex PCR assay.

Materials and methods

Samples

During a 4-month period, samples of sheep yogurt (n=40) and cheese (n=40) were purchased randomly from supermarkets in Ahvaz, South-West of Iran. According to the information provided by the vendors, all samples contained pure sheep's milk. The samples were placed in the cold portable insulated boxes, transported to the laboratory, and after that the next analysis steps were immediately began.

DNA extraction

To extract DNA from yogurt samples, 18 ml EDTA (0.5 M, pH 8) and 12 ml TE buffer (10 mM Tris-HCl, and 1 mM EDTA, pH 8.0) were transferred to a 50 ml falcon tube containing 10 g yogurt sample. After mixing for 15 min, samples were centrifuged at 4000 rpm for 20 min at room temperature. Supernatant, including the hardened fat layer and the aqueous middle phase were discarded and 1 ml lysis buffer (100 mM Tris-HCl pH 8, 200 mM NaCl, 0.1% SDS, 1% Triton X-100, 5 mM EDTA pH 8) and 15 µl Proteinase K (20 mg/ml) were added to the remaining pellet. After overnight incubation at 55 °C, the lysate was used for the usual stepwise method of DNA extraction with phenol: chloroform: isoamylalcohol (25:24:1) and finally precipitated with one-tenth volume of sodium acetate (3 M, pH 5.2) and 2.5 volume of chilled absolute ethanol. The precipitated DNA was washed in 80% alcohol, dried and dissolved in 50 µl sterile distilled water (Branciari et al., 2000; Murphy et al., 2002). To extract DNA from cheese samples, 10 ml lysis buffer (100 mM Tris-HCl pH 8, 200 mM NaCl, 0.1% SDS, 1% Triton X-100, 5 mM EDTA pH 8) and 50 µl Proteinase K (20 mg/ml) were transferred to a 50 ml falcon tube containing 5 g cheese sample and incubated overnight at 55 °C. Subsequently, the lysate was used for the usual stepwise method of DNA extraction with phenol:chloroform:isoamylalcohol (25:24:1) and ethanol precipitation as mentioned for the yogurt samples. The extracted DNA of all samples and control ones were evaluated qualitatively and frozen until PCR analysis (Branciari et al., 2000; Murphy et al., 2002).

Multiplex PCR assay

Oligonucleotide primers used for multiplex PCR assay are listed in Table 1. Amplification condition for multiplex PCR was as follows: 3 min at 94 °C; 35 cycles of 45 s at 94 °C, 45 s at 60 °C, and 1 min at 72 °C; and a final 5-min extension at 72 °C. The presence of the 279 bp cow-specific, 157 bp goat-specific and 331 bp sheep-specific amplification products were checked on 1.5% agarose gel (Bottero et al., 2002; Matsunaga et al., 1999).

Determination of the detection limit

A reconstruction experiment was carried out to determine the detection limit of the assay. Samples of sheep yogurt and cheese containing 0.5, 1, 2, 4, 6, 8 and 10% (v/v) of each of the cow's and goat's milk were prepared. These mixed samples were then subjected to DNA extraction and subsequent multiplex PCR. Finally, the detection limit of the method was estimated by agarose gel electrophoresis of the PCR products.

Statistical analysis

SPSS, Inc, Chicago, IL software (v. 16.0) was used to compare the percentages of different fraudulent practices among yogurt and cheese samples by Fischer's Exact as well as Chi-square tests. *P* values of less than 0.05 were considered statistically significant.

Results

In the present study, a multiplex PCR assay was used to detect the fraudulent addition of cow's or goat's milk to the sheep products. The extraction method used to isolate DNA from yogurt and cheese samples showed good DNA yield and quality. As shown in Fig. 1, simplex and

multiplex PCR assays on extracted DNA from mixed samples of yogurt and cheese, resulted in 157, 279 and 331 bp amplification products for goat, cow and sheep, respectively. Results of the reconstruction experiments to determine the detection limit of the method are presented in Fig. 2. As indicated, the limit of detection of cow's or goat's milk in sheep yogurt was 2%, while this limit was 4% in sheep cheese.

Table 2 shows the results of PCR analyses of sheep yogurt and cheese samples from retail trade. Out of all samples, only 27.5% of the yogurt samples and 20% of the cheese samples contained pure sheep milk (p>0.05). In 12.5% of the yogurt samples and 17.5% of the cheese samples, no apparent sheep-related amplification product was observed, suggested that cow's or goat's milk were entirely substituted for sheep's milk in these samples. Interestingly, 4 (10%) of the cheese samples contained all three types of milk.

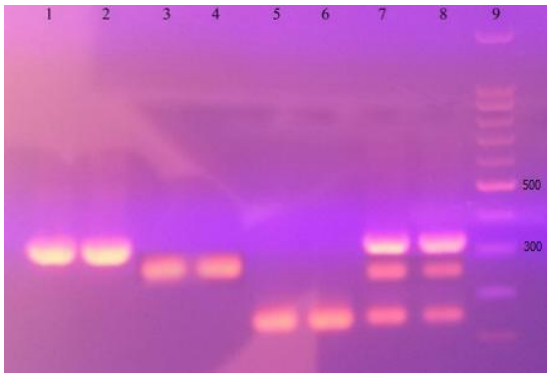

Fig. 1: Results of simplex and multiplex PCR assays on extracted DNA from ternary mixture samples of yogurt (lanes 1, 3, 5, 7) and cheese (lanes 2, 4, 6, 8). Lanes 1-2: simplex PCR by sheep-specific primers (331 bp); lanes 3-4: simplex PCR by cow-specific primers (279 bp); lanes 5-6: simplex PCR by goat-specific primers (157 bp); lanes 7-8: multiplex PCR; lane 9: 100 bp DNA ladder

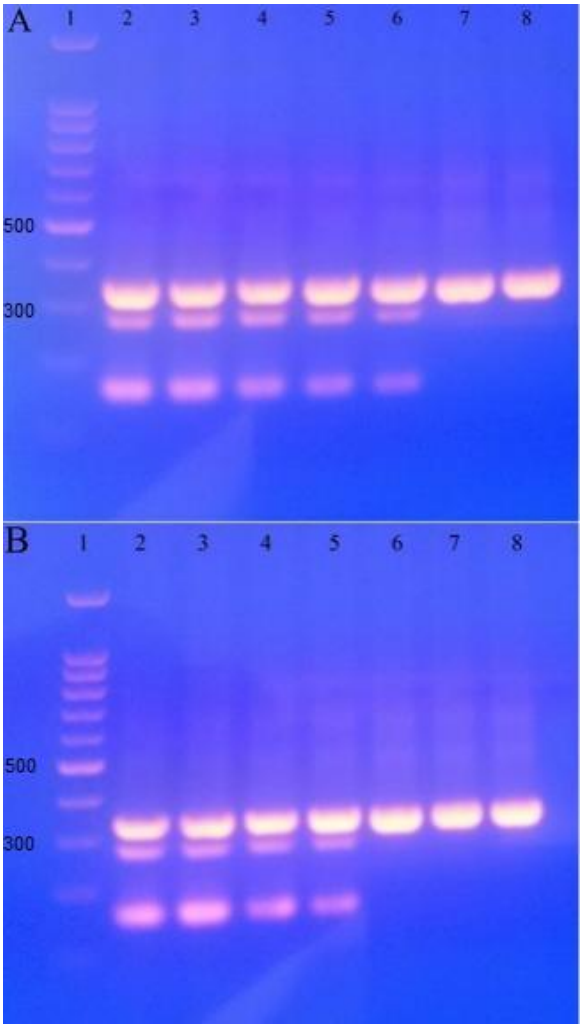

Fig. 2: The limit of detection of cow's and goat's milk in sheep yogurt (A) and cheese (B). Lane 1: 100 bp DNA ladder; lane 2: 10% cow's and goat's milk; lane 3: 8% cow's and goat's milk; lane 4: 6% cow's and goat's milk; lane 5: 4% cow's and goat's milk; lane 6: 2% cow's and goat's milk; lane 7: 1% cow's and goat's milk; lane 8: 0.5% cow's and goat's milk

Table 1: Oligonucleotide primers used in this study

Species	Primer sequence	Target gene	Product size	References
Cow	Forward: 5′-GGCTTATATTACGGGTCTTACACT-3′ Reverse: 5′-GGCAATTGCTATGATGATAAATGGA-3′	cyt b	279 bp	Bottero et al. (2002)
Sheep	Forward: 5′-GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA-3′ Reverse: 5′-CTATGAATGCTGTGGCTATTGTCGCA-3′	cyt b	331 bp	Matsunaga et al. (1999)
Goat	Forward: 5′-GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA-3′ Reverse: 5′-CTCGACAAATGTGAGTTACAGAGGGA-3′	cyt b	157 bp	Matsunaga et al. (1999)

Table 2: Species analyses of labeled sheep yogurt and cheese samples using PCR

Sample	Total No.	Pure sheep milk	Pure cow milk	Pure goat milk	Sheep and cow milk	Sheep and goat milk	Sheep, goat and cow milk
Yogurt	40	11 (27.5%)	2 (5%)	3 (7.5%)	15 (37.5%)	9 (22.5%)	0 (0%)
Cheese	40	8 (20%)	5 (12.5%)	2 (5%)	14 (35%)	7 (17.5%)	4 (10%)
Total	80	19 (47.5%)	7 (17.5%)	5 (12.5%)	29 (72.5%)	16 (40%)	4 (10%)

Discussion

In the present study, only 27.5% of the yogurt samples and 20% of the cheese samples contained pure sheep milk that shows the species adulteration in milk products is common in this region of Iran. Previously, species adulteration in milk samples marketed in another province of this country has been found by Khanzadi et al. (2013) who reported that only 21 out of 105 (20%) samples of sheep's milk in Mashhad, North-East of Iran contained pure sheep's milk and undeclared presence of cow's and goat's milk were detected in 33 (31.5%) and 68 (65%) of the samples, respectively. There are some other similar reports published previously indicate that species adulteration in dairy products may be occurred in all over the world. In Romania, the presence of undeclared cow's milk was detected in 67.3% of goat and sheep cheeses (Stanciuc and Rapeanu, 2010). Maskova and Paulickova (2006) found undeclared presence of cow's milk in 3 out of 17 goat cheeses and 1 out of 7 sheep cheese samples marketed in the Czech Republic. Di Pinto et al. (2004) evaluated 30 samples of mozzarella cheese in Italy which 22 of them contained cow's milk. Santos et al. (2003) analyzed 13 cow, goat, and sheep cheeses declared as pure in Portugal which 4 out of samples had undeclared constituent. In another study in Portugal, Mafra et al. (2004) found that only 8 out of 10 ovine cheeses purchased and analyzed contained the species ingredients as listed on the package.

According to the above mentioned researches, species identification in milk as well as dairy products has received special attention in last due to high fraudulent in this matter. In many countries, based on national laws, producers must clarify the type of milk used for manufacturing dairy products. Species identification of milk and dairy products is important for several reasons relating to public health, religion, trade, and government regulations. To assure consumers of accurate labeling, it is necessary to prove the authenticity of labels, using fast, reliable and sensitive methods for species identification. PCR is the most widely used molecular technique for the identification of the species of origin in food, especially in meat products (Ghovvati et al., 2009; Haunshi et al., 2009; Kesmen et al., 2007; Mehdizadeh et al., 2014; Rodriguez et al., 2003; Rodriguez et al., 2004). In contrast, application of PCR-based techniques to the authentication of dairy products has been very limited. The detection limit of the multiplex PCR method used in this study was 2%, for detection of cow's or goat's milk in sheep yogurt; and 4%, for detection of cow's or goat's milk in sheep cheese. Since non-authentic dairy products are produced in order to financial gain, adulterating a more expensive type of milk with a less costly type for less than 5% lacks any economic gain and therefore is unlikely (Khanzadi et al., 2013; Maudet and Taberlet, 2001). So, the determined detection limits of the multiplex PCR assays described herein are sufficient for the proof of undeclared component of the products.

Conclusion

We found high level of adulteration in the retail trade of sheep dairy products. Therefore, careful and continual surveillance on the production and sale of these products should be considered. In order to conduct a detailed monitoring, it is necessary to have a fast and accurate diagnostic technique. The multiplex PCR method used in this study is a useful and straightforward approach for the detection of low levels of cow's or goat's milk in sheep products. So, to avoid unfair competition and to assure consumers of accurate labeling, this method can be recommended to the regulatory agencies.

Conflicts of interest

There is no conflict of interest in this study.

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