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Application of triplex-PCR with an innovative combination of 3 pairs of primers for the detection of milk's animal origin in cheese and yoghurt

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Research Article

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Abstract

In this research communication we describe an innovative protocol that combines three pairs of primers, two from the literature and one designed in our laboratory, for application in triplex-PCR on somatic cell DNA to enable identification of the species origin (cow, sheep, goat) of cheeses and yogurts with a detection limit of 0.1%. Mislabeling was detected in 15 out of 40 cheeses and in 18 out of 40 yogurts tested. The suggested procedure is a quick and reliable tool for identifying the animal origin of cheeses and yogurts and it can be used to certify product reliability on the domestic and international market. Additionally, in combination with a serological test it can offer a reliable tool for detecting the presence of cow's whey.

Identification of milk origin in dairy products is of great importance because milk is associated with allergic reactions and, possibly, metabolic diseases (Drummond *et al.*, 2013). Also, substitution of milk of another species may result in economic fraud (Maščová and Paulíčová, 2006). Mislabeling of products particularly in Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) is a violation of European and National law relating to the labeling of food (Araújo *et al.*, 2016). There is an urgent need for fast and accurate analytical methods for investigating food quality, especially when it is subjected to processing such as cheese and yoghurt. Reliable, fast, sensitive and reproducible methods are based on DNA analysis of somatic cells contained in milk, since DNA does not depend on breed, age or lactation stage of the animal. The identification of milk's and cheese's animal origin by PCR was first reported by Plath *et al.* (1997) followed by many others (Mašková and Paulíčová, 2006; Golinelli *et al.*, 2014). Multiplex PCR methods can detect simultaneously cow, goat and sheep milk in cheese and yoghurt (Bottero *et al.*, 2003; Zarei *et al.*, 2016).

The purpose of this study was to develop a method of very high sensitivity, accuracy, speed and of low cost able to detect and identify easily the animal origin of milk (cow, goat, sheep) contained in Greek cheeses and yoghurts.

Materials and methods

Sampling

One to three lots from 40 different types of local cheeses were analyzed from various dairies of Greece. They were classified in six groups according to milk's origin indicated on the label: cow, sheep, goat, mix of cow-sheep, mix of goat-sheep and mix of cow-goat-sheep (Table 1).

One to three lots from 40 different yoghurts of industrial origin were selected in Northern Greece. Within these samples were included yoghurts labeled as cow, sheep or goat according to Table 1.

DNA extraction

Somatic cells' DNA of cheeses and yoghurts were extracted (PureLink Genomic DNA Extraction Kit; Thermo Fisher Scientific, USA). The yoghurt samples (5 ml each) were initially incubated at 60°C for 2 h with the kit's digestion buffer and proteinase K due to the high content of proteins in yoghurt. The cheese samples (10 g each) were firstly homogenized with 90 ml of sodium citrate (2%) for 3 min. Both yoghurt and cheese samples were treated with sodium citrate (2%) and successive centrifugations.

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Table 1. Origin of milk according to label at the 40 samples of cheeses and yoghurts used in the study

Origin of milk according to label	Number of cheese samples	Number of yoghurt samples
Cow	13	25
Goat	6	5
Sheep	5	10
Goat and sheep	5	–
Cow and sheep	1	–
Cow, goat and sheep	10	–
Total	40	40

(F: 5′-CGC TCG CCT ACA CAC AAA TA-3′ and R: 5′-CGT GCT TAA TAT GCA TGT GG-3′) designed in our laboratory on *Capra hircus* mitochondrion genome (MK234705.1, nt 226–460), by using the Primer3 Plus Software, amplifying a 234 bp of D-loop mtDNA fragment, were checked in triplex-PCR. The Bovine primers (Lahiff *et al.*, 2001), the Ovis (Bottero *et al.*, 2003) and the one we designed for caprine, showed the highest specificity. PCR reactions were performed in a final volume of 10 µl, containing 1× KAPA 2G Multiplex PCR Mix (KAPA Biosystems), 300 nM of each primer and 80–100 ng DNA. The thermocycler protocol included an initial denaturation step for 3 min at 95°C followed by 30 cycles of: 95°C for 15 s, 60°C for 30 s and 72°C for 30 s and the final elongation at 72°C for 7 min.

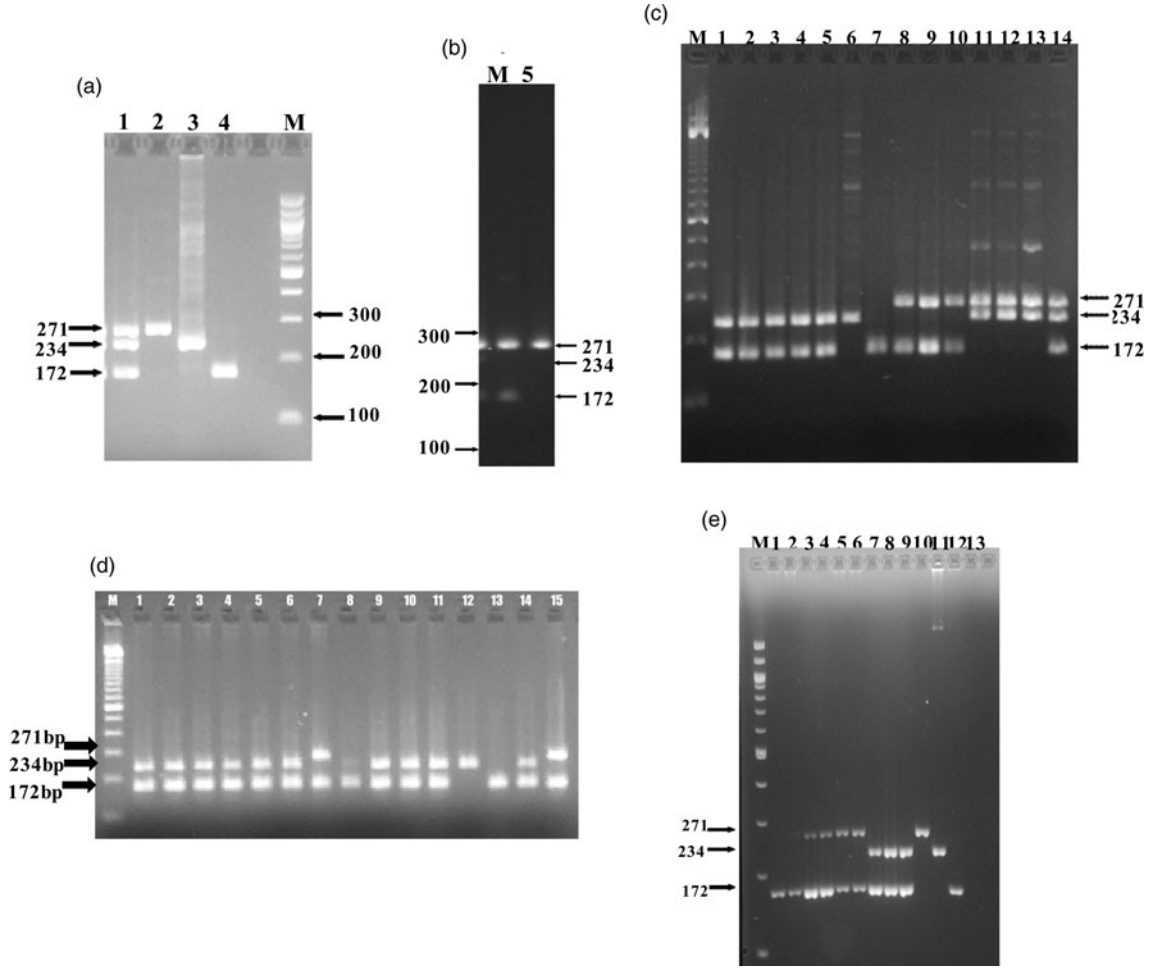

Fig. 1. Electrophoresis on 2.5% agarose gels of triplex-PCR products from analysis of (a): cheese, (b): yoghurt. M:100 bp DNA ladder (Invitrogen). 1: cheese, 2–4: controls (three species' DNA), 5: yoghurt. (c and d) products of the ring test. (c) 1–7: mix of sheep and goat milk (percent 90–10, 80–20, 60–40, 30–70, 50–50, 100–0, 0–100), 8–10: mix of cow and sheep milk (percent 50–50, 30–70, 70–30), 11–13: mix of cow and goat milk (percent 50–50, 30–70, 70–30) 14: mixture of cow, sheep and goat milk (ratio 1 : 1:1). (d) 1–6: mix of sheep and goat milk (percent 90–10, 95–5, 98–2, 99–1, 92–8, 88–12), 7: 90% sheep and 10% cow, 8–14: mix of sheep and goat (percent 80–20, 78–22, 75–25, 70–30, 0–100, 100–0, 85–15) and 15: 75% sheep and 25% cow milk. (e) Triplex PCR assay sensitivity. 1–6: sheep milk contaminated by cow milk (percentage of cow milk: 0.05, 0.1, 0.3, 0.5, 1, and 2%), 7–9: sheep milk contaminated by goat milk (percentage of goat milk 0.1, 0.3, and 0.5%), 10–13: control (DNA of the three species).

Triplex-PCR

A series of eight pairs of primers, published by other investigators, (Lahiff *et al.*, 2001; Bottero *et al.*, 2003; Mašková and Paulíčová, 2006; Golinelli *et al.*, 2014; Agrimonti *et al.*, 2015) and one

For the validation of the method and the determination of its sensitivity a ring test trial was carried out on milk samples with contaminations of cow, sheep and goat milk (percentage ranging from 0.05 to 70%).

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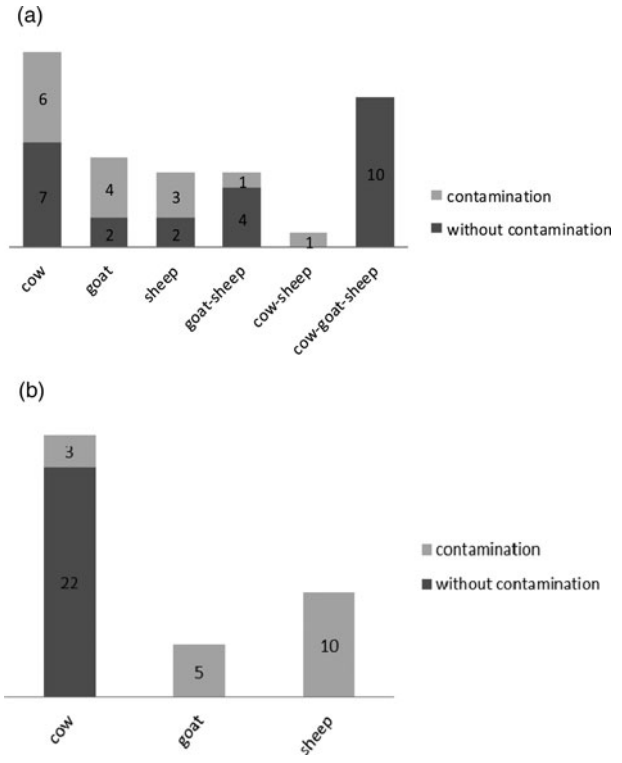

Fig. 2. Determination of animal origin of milk in: (a) 40 cheese and (b) 40 yoghurt samples. On the horizontal axis the cheese's or yoghurt's milk origin is indicated according to the label, while on each histogram there is the number of cheeses and yoghurts with or without contamination.

As reference samples we used DNA extracted from milk and blood samples collected by veterinarians from the three species.

Serological test

The Rapid Test Cow kit (Prognosis Biotech, Hellas), for bovine IgG detection, was applied on 7 cheese samples (3 goat, 3 sheep and one sheep-goat) and 12 (6 sheep and 6 goat) yoghurt samples. The results were validated with samples positive and/or negative for cow's DNA according to PCR.

Results

The triplex PCR assay proposed in this study was validated by the ring test as described in materials and methods (Fig. 1c and d). Its sensitivity was determined to be 0.1% of contamination (Fig. 1e).

Undeclared milk was detected in 15 out of the 40 cheeses (37.5%) and in 18 out of the 40 yoghurts (45%). In Figure 1a we show the results regarding the analysis of a cheese sample labeled as cow and sheep by triplex-PCR, where a third band (234 bp) corresponding to goat mtDNA was observed in addition to the expected ones (271 and 172 bp). Figure 1b shows the presence of milk of all three species as identified by triplex-PCR in one yoghurt sample.

Not labeled goat and/or sheep milk were detected in almost 50% of the cheeses labeled as cow. In most of the goat products we detected sheep and/or cow or goat and/or cow milk, respectively. Cow milk was detected in only one cheese labeled as mixture of sheep and goat milk, while a cheese labeled as cow and sheep cheese was found to contain goat milk as well (Fig. 2a).

In regard to the yoghurts (Fig. 2b), in 25 labeled as cow, 22 contained only cow milk while the rest 3 contained milk of different origin, (2 contained all three species' DNA and 1 sheep's DNA). Undeclared milk was detected in all of the 10 yoghurts labeled as sheep (6 contained both cow's and goat's DNA, 2 cow's DNA and 1 goat's DNA). Undeclared milk was also found in all the five yoghurts labeled as goat, (3 contained cow's and sheep's DNA and 2 only sheep's DNA).

Application of the Rapid Test Cow kit (RTC) showed similar results to those of PCR. In only one cheese, where PCR did not detect cow milk, bovine IgG was indicated. In 8 out of the 12 yoghurt samples labeled as sheep and goat, the results of PCR and RTC were the same. However, in two cases the kit did not detect bovine IgG although PCR was positive for cow's DNA, while in other two the opposite was found, (PCR did not detect cow's DNA while bovine IgG was present).

Discussion

The mislabeling in Greek dairy products is of great national concern, as both Greek cheese and yoghurt are important export products. It is, therefore, important to develop accurate, sensitive, fast and effective methods for the detection of milk origin of dairy products.

The detection limit of the suggested method was determined to be 0.1% (Fig. 1e) which is considered very satisfactory in order to ensure consumer protection and provide added value to the tested cheeses and yoghurts. According to the Commission Regulation (EC) No. 273/2008 in refer to cow milk, undeclared milk ≥1% is considered as illegal although Maščová and Paulíčová (2006), suggested detection limit of 5% as sufficient for the proof of undeclared milk component, since adulteration up to that level lacks of any economic effect.

Our methodology indicated the presence of milk of different origin in 15/40 cheese samples and in 18/40 yogurt samples tested. In general, cow milk was detected only in a small number of goat or sheep cheese samples as a third addition while the presence of unlabeled sheep or goat milk was more frequent in products labeled as of pure cow origin. Of the 15 goat and sheep yoghurts, only in two sheep yoghurts was contamination with cow milk detected. The contamination of cow cheeses and yoghurts with goat and/or sheep milk cannot be considered as an economic fraud for the consumer, since cow milk is cheaper than the goat or sheep milk. It is more likely this contamination is either due to the use of the same manufacturing equipment to produce cheese or yoghurt with milk of different animal origin or due to the fact that in Greek farms, those that focus on one species (goat or sheep) it is nevertheless quite common to accommodate a small number of the other species and collect the milk in the same milk cooling-tank. This may lead to the detection of undeclared milk by the PCR due to the very high sensitivity of this technique.

The partial evaluation of our method with the serological kit detecting bovine IgG immunoglobulins reassured its credibility. Only one cheese and four yoghurts showed a discrepancy in the results and this may suggest the presence of cows' whey where all seroproteins, IgG included, are contained but no somatic cells. If this is the case the combination of our methodology with the kit could be a very useful and reliable tool also for the detection of cow serum in dairy products.

In conclusion, the suggested methodology is specific and reliable for determining the origin of the milk used for the production of cheeses and yoghurts. It could, therefore, be applied by official laboratories to provide a certificate that will enhance the

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product's reliability on the domestic and international market. Since contamination with milk of different origin than the labeled may be due to either mixed farming or inappropriate procedures it is important to make the necessary recommendations to the appropriate authorities to ensure the protection of the consumer and the producer.

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