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A simple and fast triplex-PCR for the identification of milk's animal origin in Halloumi cheese and yoghurt

Evdokia Kastanos[1], Christos Papaneophytou[2], Thanasis Georgiou[2] and Catherine Demoliou[2]

[1]Department of Biology, Montgomery College, 51 Mannakee St, Rockville, MD 20850, USA and [2]Department of Life and Health Sciences, University of Nicosia, School of Sciences and Engineering, 2417, Nicosia, Cyprus

Research Article

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Author for correspondence:
Christos Papaneophytou,
Email: papaneophytou.c@unic.ac.cy

Abstract

In this research communication we describe a straightforward triplex-PCR protocol able to differentiate the origin of milk from three closely related species (goat, sheep and cow) in Halloumi, a cheese with Protected Designation of Origin (PDO), and yogurts. Halloumi must contain at least 51% sheep or goat milk, therefore, the fraudulent adulteration of this cheese with excess of cow milk must be routinely tested. The assay employs one universal forward primer and three species-specific reverse primers giving rise to 287 bp (cow), 313 bp (goat), and 336 bp (sheep) amplicons, under the same amplification conditions. This protocol, when used to test a small number of Cyprus commercial products, correctly detected mislabeling in Halloumi (2 out of 6 samples were adulterated) and yogurt brands (1 out of 4 was adulterated). The suggested protocol is a reliable tool for identifying the origin of milk in Halloumi cheeses and yogurts and can be used in any laboratory equipped with a thermocycler and an agarose gel electrophoresis apparatus.

Assessment of the authenticity of dairy products is an important issue, not only due to the economic point of view, but also due to consumer medical requirements (e.g. allergies) or religious practices (Ortea *et al.*, 2016). To this end, European and global food regulations have introduced the 'Farm to Fork' strategy which implies the traceability and authentication of the product from raw material (farm) to consumer (fork) (https://ec.europa.eu/food/horizontal-topics/farm-fork-strategy_en).

Halloumi/Hellim is a rindless semi-hard cheese that was traditionally made in Cyprus using fresh sheep or goat milk or a mixture of both (Papademas and Robinson, 1998). In 2012 a minimum of 51% milk from sheep and goats became mandatory according to the Official Gazette of the Republic of Cyprus (30 Nov 2012, issue No 4628, p. 4787) (Tarapoulouzi *et al.*, 2020). Moreover, Halloumi cheese was registered in March 2021, by the EU as a protected designation of origin (PDO) Cyprus product (https://eur-lex.europa.eu/eli/reg_impl/2021/591/oj). However, local small Halloumi producers are concerned about economic fraud because of adulterations with more than 49% cow's milk, which is in surplus in the market. Therefore, the development of powerful analytical methods to detect any milk adulteration in dairy products, including Halloumi cheese, is considered very important in food authentication (Amaral, 2021). Several analytical techniques involving sensory, chromatographic, protein electrophoresis, spectrophotometric and DNA-based as well as physicochemical methods have been used for species origin identification of milk in food category products (Hong *et al.*, 2017; Trimboli *et al.*, 2019). Species identification of milk used in Halloumi cheese production has been reported using protein-based methodologies (casein: Moatsou, 2004; Recio *et al.*, 2004). The most recent method developed to identify the species origin of milk in Halloumi cheese involved chemometric analysis that required Fourier transform infrared spectroscopy (FTIR) and complex computer software (Tarapoulouzi *et al.*, 2020). However, PCR-based methods to identify the species origin of milk in Halloumi cheese have not yet been developed.

The aim of this study was the development of a highly specific, sensitive and fast assay using PCR for the qualitative assessment of the species origin of milk in Halloumi cheese. Such a method could be used for identifying adulterations in other dairy products and further developed into a specific assay kit for milk authentication in Halloumi cheese production.

Materials and methods

Sample testing

Animal blood samples (3–5 ml) were obtained from an equal number of animal breeds of either cow (*Bos taurus*: Cyprus and Freezer) or goat (*Capra hircus:* Damascus (D) and D ×

Alpine, Cyprus × D and Sannes × D crosses) or sheep (*Ovis aries:* Cyprus Fat-tailed (L), Cyprus type Chios (C), Awassi (A) and crosses (C × L, A × C, and C × Fresian), with the cooperation of the Veterinary Department of the Cyprus Ministry of Agriculture, Rural Development and Environment and as part of routine veterinary diagnostic examination, not requiring ethical approval. Blood samples were pooled and 1 ml was subsequently used for DNA isolation. Pooling of breed blood samples was undertaken because Cyprus producers of dairy products use species-specific pooled milk collected at each farm, irrespective of breed.

Raw milk from the three animal species (cow, goat, and sheep) including their breeds, was collected under specified hygienic conditions from local farms. Milk samples from breeds were also pooled and used to prepare mixtures of milk containing different amounts of milk from one or more species, in various percentages. The raw milk samples and mixtures thereof were either stored directly at −20 °C or pasteurized at 90 °C for 20 min before storing.

Two lots of yogurt from four Cyprus local producers of dairy products were collected randomly from local supermarkets and classified according to the species origin of milk stated in their marketing labels by the producer as being either 100% of cow or sheep milk or 50% goat + 50% sheep milk. Samples (1.0 mL) removed under sterile conditions from the middle of each yogurt container were stored at −20 °C for DNA isolation.

Two lots of Halloumi cheese made by the same producers (6 brands) were also collected. These, according to their marketing labels, were made either from 100% goat milk or from 50% goat + 50% sheep milk. Blind Halloumi cheese samples were prepared by local producers upon our request using specified mixtures of either sheep + cow milk or goat + cow milk. Samples of 1.0 g from Halloumi cheese were removed from the middle of each piece (after unfolding the cheese), and stored at −20 °C, for DNA isolation. Both Halloumi and yogurt samples obtained from supermarkets were coded to protect the anonymity of the producer.

DNA extraction

Total DNA from 1.0 mL mixed breed blood was isolated using the Nucleospin DX blood kit (Macherey-Nagel, Germany) according to the instructions provided by the manufacturer. Total DNA from milk (1.0 ml) samples of halloumi cheeses (1.0 g) and yogurts (2.0 g) was isolated using the DNeasy Mericon Food Kit (Qiagen) according to the instructions provided by the manufacturer. Halloumi cheese samples were first minced and then homogenized (2 × 10 s) using the Powergene 125 (Fisher Scientific) homogenizer in 15 ml of 10 mM Tris-HCl, pH 8.0 containing 5 M guanidine hydrochloride, 2 mM EDTA, 150 mM NaCl, 1% (w/v) SDS, and 0.5% (v/v) Triton X-100. Total DNA was isolated after proteinase K (80 µg/ml) treatment under shaking (60.0 °C, 18 h), followed by precipitation using chloroform-ethanol (López-Calleja *et al.*, 2004). The collected DNA was stored in ddH_2O at −20 °C. DNA extraction from Halloumi cheese gave a large pellet after precipitation of DNA, most likely as the result of the high amount of salt used during the preparation of cheese. DNA samples extracted from Halloumi cheese were further purified using a spin column (Qiagen).

Triplex PCR

Primers for PCR-amplification of species-specific sequences of the mitochondrial cytochrome *b* (*cyt b*) gene were designed according to Tobe and Linacre (2008), from the gene sequences of cow (NC_0068530), sheep (NC_001941), and goat (NC_005044), and custom- made by MWG Operon (Germany). One universal forward (UN-FW, 5′ TGAGGACAAATATCATTYTGAGGRGC 3′) and three species-specific reverse primers were used: *Bos taurus* (BO-RV), 5′ TAAGATGTCCTTAATGGTATAGTAG 3′, *Capra hircus* (CA-RV), 5′ TTAGAACAAGAATTAGTAGCAT GGCG 3′, and *Ovis aries* (OV-RV), 5′ GGCGTGAATAGTACT AGTAGCATGAGGATGA 3′. The properties of the primers are summarized in online Supplementary Table S1.

DNA amplification by PCR was performed in a final volume of 20 µl containing 20 mM Tris-HCl, (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs mix, 0.5–1 pmol from each primer, 2.0 ng DNA, and 1.5 units of Taq DNA polymerase (Invitrogen). The thermocycler protocol included a denaturation step for 5 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72°C for 45 s, and a final step at 72°C for 20 min. For positive control, we used DNA derived from pooled blood samples of cow, goat, or sheep breeds. For negative control and detection of any contamination, we replaced DNA with ddH_2O in the assay. We visualized the PCR products with ethidium bromide (0.4 µg/ml) after electrophoresis on 3% agarose gels, in Tris Borate EDTA (TBE) buffer. Molecular sizing of amplified fragments was based on DNA ladders used (NIPPON Genetics).

Results

Assay validation

The triplex PCR assay proposed in this study was initially validated using DNA extracted from pooled blood samples of breeds of cow, goat, or sheep used in Cyprus, as well as from binary mixtures of sheep with cow blood, and sheep with goat blood in various amounts. The system positively detected the presence of target species (Fig. 1a), and as shown, the size of the target amplicons obtained was approximately 287 bp for cow, 313 bp for goat, and 336 bp for sheep. Despite pooled blood samples of the same species used for DNA extraction, only one amplified fragment of the expected size for the corresponding species was observed when tested using triplex-PCR. The intensities of the DNA bands in the samples of sheep blood mixed with an increasing amount of cow blood indicated that the assay system could detect contamination of ≥1% cow blood (Fig. 1a; Lane 99 : 1).

Subsequently, the assay was validated further using DNA extracted from raw cow milk as well as from mixtures of raw milk samples (Fig. 1b and c). Triplex PCR assays on extracted DNA from mixed milk samples, resulted in the amplification of the corresponding species-specific DNA fragment expected based on the origin of milk used in the mix. The sizes of these amplicons (287, 313, and 336 bp) corresponded to those seen in blood samples from cow, goat, or sheep, respectively (Fig. 1b). As shown in Fig. 1c, the detection sensitivity of the assay for sheep milk in binary mixtures of cow milk containing increasing % of sheep milk, was also in the same range as that observed for cow (≥ 1%) with blood DNA (Fig. 1a). Similarly, the obtained profile of the amplified DNA fragments, from DNA extracted from Halloumi cheese made to specifications with regards to the content of species origin of milk used (Fig. 1d), indicated that the triplex PCR assay can be used effectively to detect the species origin of any undeclared milk when added in > 1.0% amounts during the making of Halloumi cheese.

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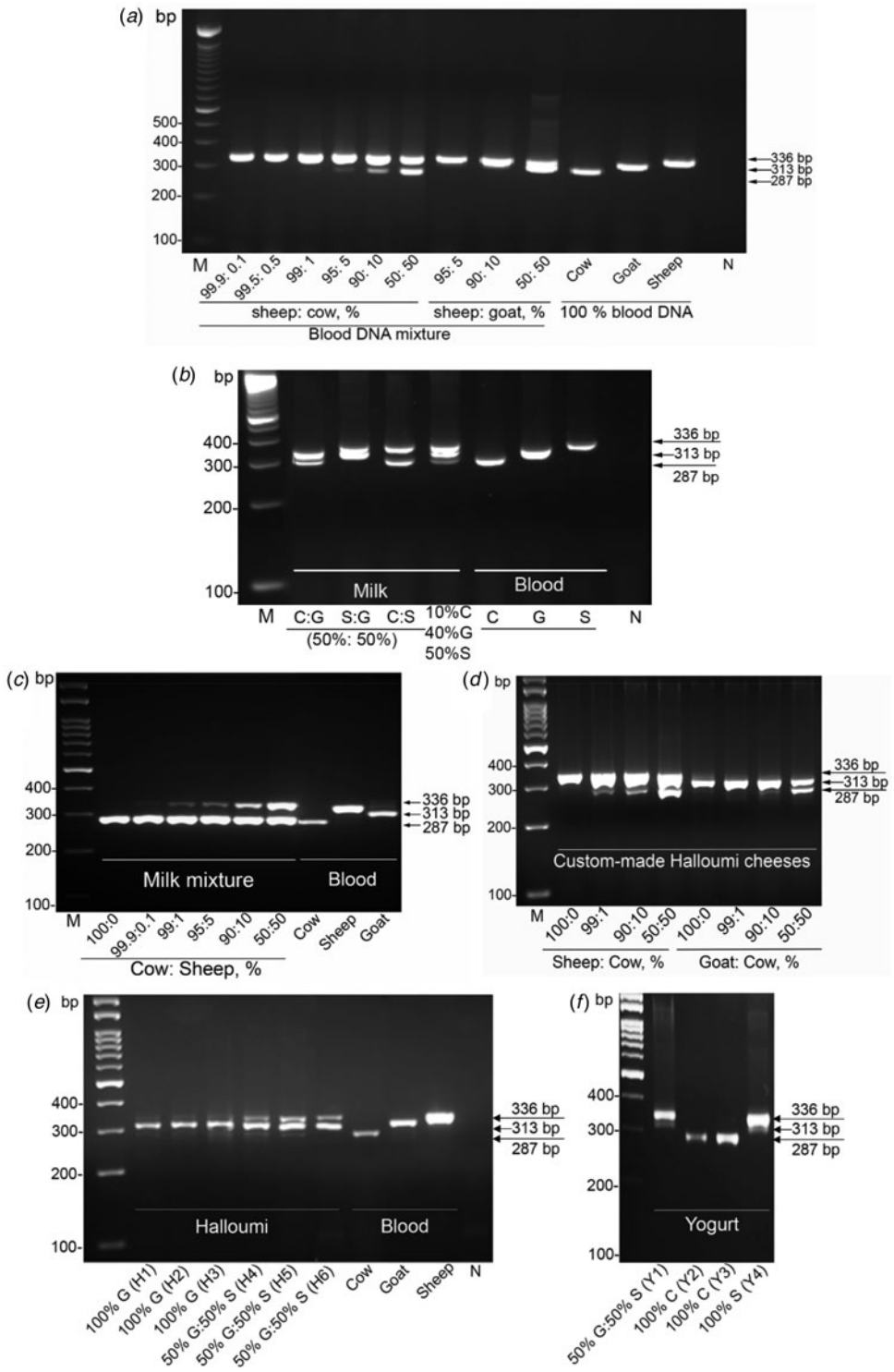

Fig. 1. Electrophoresis on 3% agarose gels of the triplex PCR-amplified fragments obtained from DNA extracted from (a) binary mixtures of sheep blood containing increasing amounts (%) of either cow blood or sheep blood (b) three binary milk combinations (50%:50%) using cow (C), goat (G) or sheep (S) milk, and from DNA extracted from a mixture containing different % amounts of milk from the three species, (c) binary mixtures of cow milk samples containing increasing amounts (0% to 50%) sheep milk, (d) custom-made Halloumi cheeses using milk mixtures of sheep or goat milk containing increasing amounts (%) of cow milk, (e) commercial halloumi cheeses, and (f) commercial yogurts. In (a), (b), (c), and (e) DNA samples extracted from 100% blood from either cow, goat or sheep were used as positive controls. Lanes: M: 100-bp DNA ladder; N: negative control.

Analysis of commercially available Halloumi cheese and yogurt

Following the aforementioned validations, the triplex PCR was applied to samples of Halloumi cheese and yogurts from the retail trade, and the results are presented in Fig. 1e and f. The amounts of DNA extracted from 1.0 g of commercially available Halloumi cheese and 2.0 g of commercially available Cyprus yogurt are summarized in online Supplementary Table S2. We detected

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undeclared milk in some samples of Halloumi cheese and yogurt. Analysis of Halloumi cheese made from 100% goat milk or 50% goat + 50% sheep milk (Fig. 1e, H4 & H5) showed the presence of a third DNA amplicon of 287 bp that corresponds to that of *cyt b* sequence of the cow gene. This was also the case for one yogurt product marketed as made from 50% goat + 50% sheep milk (Fig. 1f, Y1). Other yogurt brands similar in content and type of milk or made from only sheep (100%) or only cow (100%) showed no adulteration with milk from an undeclared animal. However, the intensity of the amplified species-specific DNA bands from a number of these dairy products, when visualized on agarose gels, indicated differences in the content of species-specific milk detected compared to that stated in their labels (Fig. 1e and f).

Discussion

The mislabeling in Cyprus dairy products and especially of Halloumi cheese is of great national concern because it is an important export product. Furthermore, identifying milk species sold in the market and used in the manufacture of milk products is a critical point in the quality control measures, while according to EU regulations (No.273/2008) it is illegal to have undeclared cow milk ≥ 1.0% in dairy products. In most cases, it is impossible to identify animal species present in the product based on smell or taste. The development of analytical techniques, therefore, to assess if products are correctly labeled is sorely needed. PCR-based methods have been successfully employed for the identification of the species origin in food, especially in meat products (see Izadpanah *et al.*, 2018 and references cited therein). However, their use for the identification of dairy products is limited.

In this work, we developed a simple triplex PCR method, which distinguishes the origin of cow, sheep, and goat milk in a single reaction with a detection limit of at least 1.0%. Due to the simultaneous amplification of segments within a single PCR, this method is simple, fast and cheap. The DNA extraction method from small quantities of milk (1.0 ml), yogurt (2.0 g) and Halloumi (1.0 g) proposed is economical and gives DNA of equivalent yield and quality to that obtained from blood samples of the same animals (used as positive controls) for use in PCR. Our method identified the presence of milk of different species in 2/6 Halloumi samples and 1/4 yogurt samples. However, we consider this to be most likely due to contamination from the use of common cooling containers for milk collection from farms rather than deliberate adulteration.

PCR-based methods have been successfully used to detect the fraudulent use of other dairy products. Recently, Zarei *et al.* (2016) developed a duplex PCR to detect the fraudulent addition of cow's milk to buffalo's milk and its products. Mislabeling was detected in 70% of the milk samples, 64% of the yogurt, and 52% of the cheese samples tested. In another study, Tsirigoti *et al.* (2020) developed a triplex PCR for the detection of species origin in Greek dairy products and mislabeling was observed in 15/40 cheese and 18/40 yogurts tested.

In conclusion, the proposed method is specific and reliable and could complement and/or replace the more complex physico-chemical methods that are presently in use for the authentication of Halloumi cheese as well as in other dairy products like yogurt.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029922000577.

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