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APPLICATION OF A DUPLEX-PCR FOR DETECTION OF COWS' MILK IN GOATS' MILK

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Abstract: A duplex-PCR method, with 2 pairs of primers recognizing sequences of mitochondrial D-loop region, was developed to identify cows' milk in the milk of goats. The PCR was shown to be specific and sensitive, enabling the detection of less than 1% of cows' milk added to the milk of goats. Simultaneous use of a primer pair for goats' and cows' mitochondrial DNA fragment prevented false negative results. The method was applied to track the presence of cow DNA in goat milk available on the Polish market. A total of 54 milk samples from 3 Polish (34) and one foreign producer (20) were examined. In 33 samples, cow DNA was detected, while 21 samples, including all of the 20 samples from foreign producers, produced the goat-specific product only.

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INTRODUCTION

Milk and milk-derived products are known to be frequent causes of food-allergies. It was found that most milk proteins, even present at low concentrations, are potential allergens [19, 21]. Cows' milk is reported as the main dairy product responsible for human adverse reactions [16]. In the majority of cases it can be successfully substituted by the milk of other species, e.g. goats, without causing allergic symptoms. Thus the counterfeiting of goats' milk with cheaper cows' milk may be considered as a health risk, making species identification an important topic in current food safety requirements, thus encouraging the development of modern tools for species identification. Other problems arising from the incorporation of cheaper bovine milk to milks of higher price mainly involve government regulations and ethical or cultural objections.

The approaches developed for species identification in milk rely on capillary electrophoresis [14]; ELISA [9, 10, 18], isoelectric focusing [1], or HPLC [4, 6, 8], but the only method widely accepted as a reference tool for cows' milk detection is based on isoelectric focusing of β-casein [7].

Some methods for species identification in milk use DNA analysis. Their reliability and very low thresholds of detection make them promising as routine tools. The methods developed so far rely mostly on PCR-amplification of various regions of the mitochondrial genome. Only 2 of them assure protection from false negative results, as the mix contains primers for all the identified species in a single tube [3, 17]. Other published methods use primers for single species [2, 11, 13] or apply restriction analysis of the obtained PCR-product [5, 15].

The aim of our study was to develop a set of highly specific primers for the detection of cows' milk added to goats'

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milk, functioning in a single tube, to obtain reliable results regardless of the material analyzed, and assuring the prevention of false negative results. A second aim was the application of the method to track the counterfeit of goat milk available on the Polish market.

MATERIALS AND METHODS

Material. Samples of raw cow and goat milk obtained from local farmers were refrigerated until used. Various concentrations, from 1 to 99%, of cows' milk in goats' milk were prepared for DNA extraction and further PCR analyses. UHT goat milk was purchased in markets over a 6-month period. The sampling was carried out on the goat milk from 3 Polish producers and one producer from the European Community. The milk from a single producer was purchased in time intervals of about 2 weeks and only products with distinct lot numbers were purchased. Ten milk samples with identical lot numbers and production dates, produced within a 4.5-hour period as assessed from the product label, originated from one Polish producer.

DNA extraction. DNA from the individual samples was prepared as described by Bania *et al.* [2]. Briefly, 400 µl of milk were mixed with 200 µl of 200 mM Tris-HCl, pH 8.0 containing 100 mM EDTA, and 0.1% SDS, then 0.6 mg of proteinase K (Sigma-Aldrich, Poznań, Poland) was added. The mixture was incubated for 1 h at 55°C. The DNA was extracted by phenol and chloroform, ethanol-precipitated, and dissolved in water.

PCR conditions. The reaction mix contained 1× polymerase buffer with 1.5 mM $MgCl_2$, 20 nmole of BosD primers and 5 nmole GoaD primers (Institute of Biochemistry and Biophysics, Warsaw, Poland) (listed in Tab. 1), 200 µM of each deoxinucleotide triphosphate (Fermentas, Vilnius, Lithuania), 1U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania) and ~1 ng of DNA in a final volume of 25 µl. Thirty-five cycles at 95°C for 30 s, 52°C for 30 s and 72°C for 2 min. were performed on a T3 thermal cycler (Biometra, Göettingen, Germany). The PCR products were resolved in 2% agarose gel at 100 V and documented with a charge-coupled device camera system (Vilber-Lourmat, Marne-la-Vallée, France).

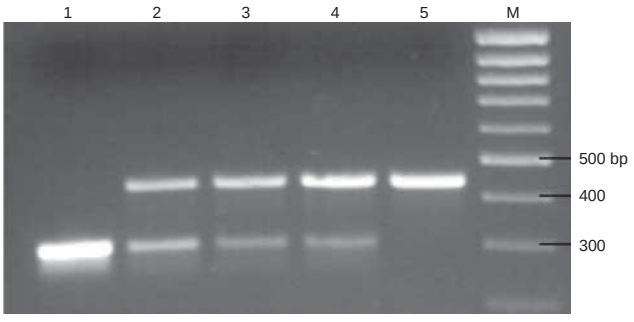

Figure 1. Agarose gel electophoresis of PCR products amplified with duplex-PCR for the fragments of cow and goat mitochondrial D-loop regions. Consecutive lanes represents the products obtained from cows' milk (lane 1) and goats' milk (lane 5). In lanes 2-4 the products obtained from mixtures of 10, 5, and 1% of cows' milk in the goats' milk were resolved. M - 100bp DNA ladder (Fermentas).

RESULTS

The comparison of the nucleotide sequences of *Bos taurus* and *Capra hircus* mitochondrial genome aligned using BioEdit software allowed the design of specific primer pairs for detection of cow and goat DNA. The specificity of the primers was evaluated on the DNA from pure cow and goat milk. Using duplex-PCR we obtained only the product of 300 bp from the cows' milk, whereas the goats' milk produced only the 444 bp band (lanes 1 and 5 in Fig. 1). The sensitivity of the assay was determined on the DNA isolated from goats' milk mixed with increasing amounts of the cows' milk. As shown in Fig. 1, the 300 bp band could be detected together with the 444 bp band even in 1% of cows' milk in goats' milk.

Fifty-four samples of UHT goat milk available on the Polish market were tested for the presence of cow DNA. Thirty-three were found to produce cow-specific PCR product in addition to goat PCR product. Twenty-one samples, of which 20 originated from a foreign producer, gave goat-specific product only.

In each of the 10 milk samples from the Polish producer differing in the hour of production, cow DNA was detected.

DISCUSSION

From a number of published approaches to detect the counterfeiting of goats' milk and its products with cows'

Table 1. Primers used in this study.

Primers	GenBank Accession No.	Location within the mitochondrial genome	Primer sequence (5' → 3' end)	Product size
BosD-for	V00654	15856-15887	CAATAACTCAACACAGAATTTGC	300 bp
BosD-rev		16135-16156	CGTGATCTAATGGTAAGGAATA	
GoaD-for	AF533441	16043-16060	CCAACATGCGTATCCCGT	444 bp
GoaD-rev		16468-16487	AGCGGATGCATGATGAAATG	

milk, only one PCR-based method, published recently by Bottero *et al.* [3], uses a set of 3 specific primer pairs allowing the detection of cow, goat, and ewe DNA in a single tube. Its advantage over the methods using a single primer pair is the elimination of false negative results.

Selection of highly polymorphic regions within the cow and goat mitochondrial D-loops, showing low homology between the 2 species, allowed us to design specific primer pairs for detection of cow and goat DNA. During the design of the primers care was taken to avoid significant Tm differences between the primers, thereby preventing the generation of unspecific products. The Tm differences of the oligonucleotides used did not exceed 0.9°C.

The duration of the elongation step was found to be important for the generation of both amplicons. Two-minute elongation allowed the detection of a 1% addition of cows' milk, while shorter elongation times significantly reduced the amount of cows' milk detected.

Previously, we tested a pair of primers designed by Matsunaga *et al.* [12] originally developed for the identification of meat mixtures of multiple species. We found that high specificity of the detection of bovine milk mixed with the milk of goat could be achieved only when specific conditions of the test, including the precise number of PCR cycles, were applied [2]. Afterwards, we designed several primers targeting various regions in the cow and goat mitochondrial genome. The tests led us to the conclusion that the primers' specificity plays a crucial role in PCR approaches for species identification in milk. Only the use of primer pairs targeting regions showing homology of less than approximately 50% between the 2 species gave satisfactory results, assuring the lack of cross-priming. The results obtained with primers recognizing sequences showing higher homology were not reliable (data not shown).

There are few published reports on the counterfeiting of goats' milk in Poland. The combination of immunological and electrophoretic methods applied in Stefaniak *et al.* [20] to examine 10 commercially available goat milk samples illustrates that the counterfeiting of this product occurs.

We examined 54 samples of UHT goat milk available on the Polish market. Twenty-one were found to produce the goat-specific product only, 20 of these samples being produced by a foreign producer. In the remaining 33 samples, cow DNA was detected.

Duplex-PCR applied in this report which is an end-point analysis, gives quantitative results only. The method is therefore unable to distinguish between milk intentionally counterfeited from cases of accidental addition of cows' milk. The latter may be due to the use of a common installation for both the cow and goat milk distribution and indicates that ineffective procedures with the equipment cleaning were applied. However, in this case, the cartons of goat milk contaminated with cow milk should constitute only a small part of a product lot. Therefore, in randomly selected goat milk samples representing the same product lot, pure goat milk together with contaminated goat milk should be detected. Our results show that 10 goat milk samples from one Polish producer differing only in the hour of production contained cow DNA. This may indicate that at least in this case, intentional fraud occurred.

Most Polish goat milk analyzed in this study was contaminated by cows' milk. Although the applied method does not allow quantification of the amount of cows' milk added, it can be assumed that some products could be counterfeited intentionally. In this case, it may contain a large amount of cow milk proteins and could be considered a health risk to individuals suffering from a food allergy. The results of the analysis of the goat milk from the foreign producer proved that its production standards are much higher.

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