PCR-Based Detection of Cow's Milk in Goat and Sheep Cheeses Marketed in the Czech Republic

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Abstract

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A method based on the polymerase chain reaction (PCR) principle was validated for detecting cow's milk in goat and sheep cheeses. DNA was isolated from the cheeses using the isolation kit Invisorb Spin Food I by Invitek Co., designed for the samples of animal origin. The PCR method applied utilizes the sequence of the mitochondrial gene coding cytochrome b which is specific for mammals. It uses the common forward primer and the reverse primer species-specific. After electrophoresis, cow DNA was characterised by the fragment of the size of 274 bp, goat DNA by the fragment of 157 bp, and sheep DNA by the fragment of 331 bp. The detection limit of the PCR method described (1%) was determined with model samples made from pure goat cheese with a defined addition of cheese made from cow's milk. The method validated was applied in the analysis of 17 goat cheeses and 7 sheep cheeses obtained from retail trade. Products of Czech, Slovak, French, Dutch, and Italian origin were examined. The presence of undeclared cow's milk was detected in three kinds of goat cheese and in one of sheep cheese.

Keywords: polymerase chain reaction; cow's milk; goat cheese; sheep cheese

The majority of retail cheeses are made from cow's milk. Pure goat and sheep cheeses are considered as specialities with characteristic sensory features, primarily the taste and flavour. Their supply fluctuates with the year season depending on the reproductive cycle of these animals. Both goat milk and sheep milk are priced much more than cow's milk. This often induces attempts to make these cheeses from raw material where goat's or sheep's milks are at least partially replaced with undeclared cow's milk. However, the consumer has the right to know the species origin of the cheese, be it for nutritional or religious reasons.

For the time being, the valid reference method for the detection of cow's milk in the dairy products is based on the isoelectric focusing of milk caseins. Other electro-migration methods, some ELISA techniques, chromatographic methods, and even a method based on the principle of mass spectrometry (Borková & Snášelová 2005) have also been tested for this purpose. The procedures mentioned above are often unsuitable for the analysis of the complex food matrix and also evince a lower sensitivity for the thermally treated substances. This is why the methods based on the polymerase chain reaction are increasingly employed in the recognition of the respective milk kinds. Some of these make use of the restriction enzyme analysis of the DNA fragment following amplification (Lipkin *et al.* 1993; Amills *et al.*

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1997; Plath *et al*. 1997; Branciari *et al*. 2000), other methods successfully focus on the mitochondrial gene coding cytochrome b (Matsunaga *et al*. 1999; Bania *et al*. 2001; Piknová *et al*. 2002).

The aim of the present paper was the validation of the PCR method exploiting the mitochondrial gene coding cytochrome b for the determination of cow's milk in goat and sheep cheeses, and the application of this method for the detection of undeclared cow's milk constituent in retail cheeses.

MATERIAL AND METHODS

The cheeses for analyses were purchased in food store chains, in bio/eco food shops, and in Italian speciality stores. They were made by 22 different producers located in five European countries. The products analysed are listed in Table 1.

DNA in cheeses originates in somatic cells, the quantity of which is strongly dependent on the season of the year, the lactation status, and particularly the clinical condition of the animal (Lipkin *et al*. 1993).

DNA was isolated from the cheeses using the isolation kit Invisorb Spin Food I (Invitek, Co., Berlin, Germany), which is designed for the food samples of animal origin and employs non-chaotropic solid phase extraction. Within the bounds of the commercially recommended isolation protocol, the conditions of DNA elution were modified to comply with the food material used. DNA was eluted from the column with 2 × 50 µl of preheated elution buffer and the eluate was kept in the refrigerator at 4°C prior to amplification. To remove the undesirable RNA from the sample, 10 mg/ml of RNAse A (Top-Bio Ltd., Prague, Czech Republic) was added during the extraction. All other conditions of the isolation protocol were strictly adhered to.

The quality and concentration of the isolated DNA were evaluated spectrophotometrically (BioPhotometer 6131, Eppendorf, Hamburg, Germany) by determining the 260/280 nm absorbance ratio (Sambrook *et al*. 1989).

The polymerase chain reaction was carried out in a cycler Touchgene Gradient (Techne, Cambridge, England). The common forward primer SIM and the reverse primer specific for the respective species were used (Matsunaga *et al*. 1999).

SIM primer: 5'GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA 3'

Cow (Cattle B): 5'CTA GAA AAG TGT AAG ACC CGT AAT ATA AG 3'

Goat (Goat G): 5'CTC GAC AAA TGT GAG TTA CAG AGG GA 3'

Sheep (Sheep S): 5'CTA TGA ATG CTG TGG CTA TTG TCG CA 3'

The reaction mixture volume of 50 µl contained:

2.5 U Platinum Taq DNA Polymerase (Invitrogen, England); 1× concentrated PCR buffer without Mg (containing 20mM Tris-HCl, pH 8.4, and 50mM KCl) (Invitrogen, England); 1.5mM $MgCl_2$ (Invitrogen, England); 0.2mM dNTP mix (Eppendorf, Hamburg, Germany); 25 pmol of each primer (Invitrogen, England); DNA extract, made up to 50 µl with sterile water Molecular Biology Grade, DNase free.

The optimised temperature and time profile of the PCR reaction was as follows: Initial denaturation: 94°C, 1 min; 40 cycles with the following step-cycle profile: denaturation 94°C, 30 s; annealing 60°C, 30 s; extension 72°C, 30 s; final extension 72°C, 5 min.

A DNA isolate from beef meat acted as a positive control of cow DNA in PCR reactions; the control DNA from goat at the concentration of 20 ng/µl (Biotools, Spain) was used for goat, and the control DNA from sheep at the same concentration for sheep.

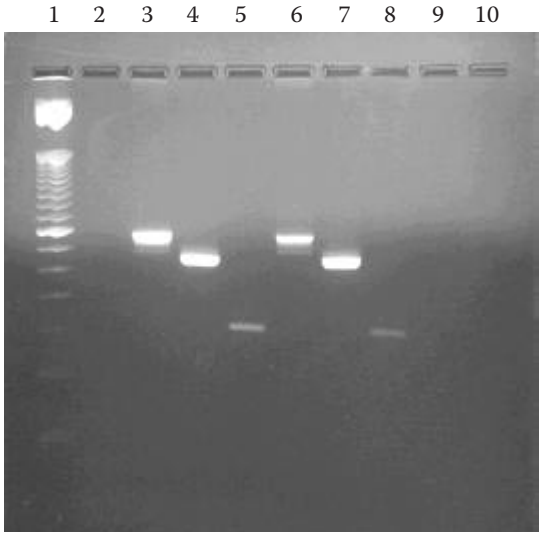
PCR products were separated by electrophoresis in 3% agarose gel in TBE buffer 1× concentrated and stained with ethidium bromide (0.5 µg/ml). Agarose gel horizontal electrophoresis was conducted at 110 V for 1 h 30 min. The standard 50 bp DNA ladder (Invitrogen, England) with a highlighted band at 350 bp was used to determine the molecular weights of the amplicons.

Visualisation was accomplished using a UV transilluminator (Herolab UVT-20, Wiesloch, Germany), and the subsequent photographic documentation with the help of a Kodak digital camera supported by Kodak 1D software.

RESULTS AND DISCUSSION

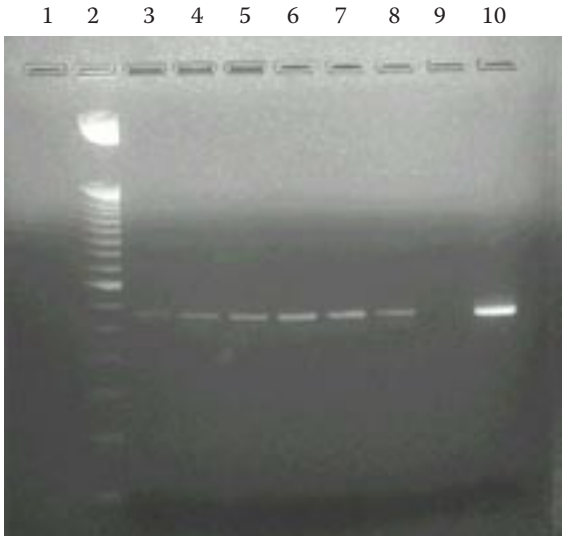
The PCR method described enabled the detection of the amplified fragments of cow DNA, size 274 bp, goat DNA, size 157 bp, and sheep DNA, size 331 bp, respectively. This is clearly shown in Figure 1.

It was necessary to determine the detection limit of this method before stating that it can



Lane 1 – 50 bp DNA Ladder (Invitrogen, England)
Lane 3 – PCR product of sheep DNA
Lane 4 – PCR product of cow DNA
Lane 5 – PCR product of goat DNA
Lane 6 – PCR product of sheep DNA
Lane 7 – PCR product of cow DNA
Lane 8 – PCR product of goat DNA
Lane 9 – negative control

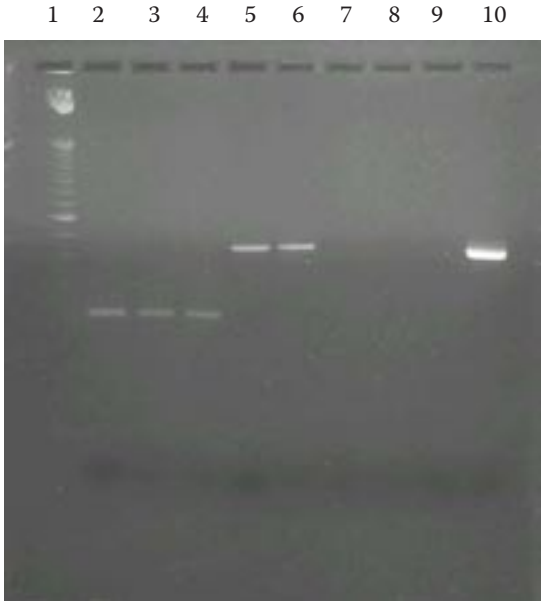
Figure 1. Detection of cow, goat, and sheep DNA (3% agarose gel, 1× TBE, 110 V, 1 h 30 min)



Lane 2 – 50 bp DNA Ladder (Invitrogen, England)
Lane 3 – 1% cow cheese in goat cheese
Lane 4 – 5% cow cheese in goat cheese
Lane 5 – 10% cow cheese in goat cheese
Lane 6 – 25% cow cheese in goat cheese
Lane 7 – 50% cow cheese in goat cheese
Lane 8 – 100% cow cheese
Lane 9 – negative control
Lane 10 – PCR product from 80 ng of beef DNA

Figure 2. Determination of the detection limit of PCR reaction (3% agarose gel, 1× TBE, 110 V, 1 h 30 min)

be reliably used for the detection of undeclared quantities of cow's milk in goat and sheep cheeses. A series of model mixtures of pure goat cheese with gervais cheese made from cow's milk was prepared for determining the detection limit. The samples were subjected to DNA isolation and amplification focused on the cow DNA detection; after electrophoresis, a 1% detection limit



Lane 1 – 50 bp DNA Ladder (Invitrogen, England)
Lane 2 – Goat standard (Biotools, Spain) – 40 ng of goat DNA
Lane 3 – Natural goat cheese with red pepper (Slovakia) – detection of goat DNA
Lane 4 – Goat brynza cheese (detection of goat DNA)
Lanes 5 and 6 – Natural goat cheese with red pepper (detection of cow DNA)
Lanes 7 and 8 – Goat brynza cheese (cow DNA)
Lane 9 – negative control
Lane 10 – PCR product from 80 ng of beef DNA

Figure 3. Example of PCR analysis of goat cheeses for the presence of cow's milk component (3% agarose gel, 1× TBE, 110 V, 1 h 30 min)

of the above-mentioned PCR reaction was found (Figure 2). This detection limit was also verified in model sample mixtures of pure sheep cheese with gervais cheese from cow's milk.

The record of an electrophoretic run capturing the undeclared cow's milk component added to goat cheese is shown in Figure 3. As clearly seen, the Slovak natural goat cheese with red pepper was unambiguously adulterated with cow's milk, in spite of being declared as pure goat cheese.

Table 1 shows the results of PCR analyses of 17 goat cheeses (Nos. 1–17) and 7 sheep cheeses (Nos. 18–24) from retail trade. Undeclared presence of cow's milk was detected in three goat cheeses (Nos. 5, 9, 10) and one sheep cheese (No. 19).

The determined 1% detection limit of the PCR reaction described was well in keeping with the results by other authors. Most research papers using the PCR technique for the estimation of undeclared additions of cow's milk give the detection limit within the range of 0.1 to 1%. Thus, the PCR method helped to detect the addition of 1% cow's milk in buffalo milk (Rea *et al.* 2001), 1.5% addition of cow's milk to mozzarella cheese (Di Pinto *et al.* 2004), or 5% addition of cow DNA to goat's, sheep's, or buffalo's milks, and 2% addition to goat cheeses (Klotz & Einspanier 2001). Plath *et al.* (1997) identified 0.5% cow's milk in goat and sheep cheeses and Feligini *et al.* (2005) detected 0.5% cow's milk in Italian mozzarella. Bottero *et al.* (2003) found various kinds of milk in dairy products by a multiplex PCR method with the same detection limit. Bania *et al.* (2001), when identifying cow's milk in goat's milk, found an even lower detection limit (0.1%), similarly as Maudet and Taberlet (2001) in goat cheeses.

Table 1. Results of analyses of goat and sheep cheeses from retail trade for the presence of cow's milk component

No.	Cheese brand or description	Country of origin	Presence of cow's milk	
			declared	detected
1	Balkan goat cheese Kaprinus	CZ	no	no
2	Fresh non-ripening natural goat cheese	CZ	no	no
3	Dessert goat cheese Doral	CZ	no	no
4	Natural goat cheese	CZ	no	no
5	Natural semi-hard sliced goat cheese Pikant	CZ	no	yes
6	Sedlák's natural goat brynza cheese	CZ	no	no
7	Sládeček's genuine semi-hard ripening goat cheese	CZ	no	no
8	Tylžský goat cheese	CZ	no	no
9	Pickled natural cheese from goat milk	SK	no	yes
10	Natural cheese from goat milk with red pepper	SK	no	yes
11	Gouda goat Polder	NL	no	no
12	Fresh natural goat cheese Chavroux	FR	no	no
13	Fresh natural goat cheese Soignon Mini Buche Blanche	FR	no	no
14	Natural goat cheese with surface mould Soignon Buchette Saint Maure	FR	no	no
15	Fresh goat cheese Cabridoux	FR	no	no
16	Goat cheese with surface mould Chevre du Poitou	FR	no	no
17	Goat cheese Tomme	FR	no	no
18	Bryndziarka	SK	yes	yes
19	Kaškaval sheep cheese	SK	no	yes
20	Liptov – full-fat winter brynza cheese	SK	yes	yes
21	Orava	SK	yes	yes
22	Slovak Feta	SK	yes	yes
23	Feta Dionys	IT	no	no
24	Pecorino Sardo	IT	no	no

The method, which is based on the mitochondrial 12S a 16 S rRNA genes, detected 0.1% addition of cow's milk in sheep's and goat's milks (López-Calleja *et al*. 2004; Mafra *et al*. 2004). Piknová *et al*. (2002) are the only authors to suggest 0.01% detection limit for the addition of cow's milk to sheep cheeses.

The European reference method of isoelectric focusing of milk caseins also has the detection limit set to 1%. Cozzolino *et al. (*2001) consider the 5% detection limit as sufficient for the proof of undeclared milk component, adding that any adulteration of less than 5% lacks any economic effect.

Similarly as in this study, some other authors conducted a survey of retail cheeses other than cow cheeses for undeclared quantities of cow's milk added. Thus Mafra *et al*. (2004) found that only 8 out of 10 cheeses purchased and analysed contained the species ingredients as listed on the package. The two remaining cheeses were made from cow's milk only, in spite of the fact that the presence of both cow's and sheep's milks was declared. The authors conclude that the development of a method quantifying sheep's milk in mixed milk cheeses is needed to prevent their adulteration. Santos *et al*. (2003) analysed 13 cow, goat, and sheep cheeses declared as pure. In four cases they detected the presence of an undeclared constituent, i.e. either cow's milk or, in two cases, goat's milk contained in "pure sheep cheese". To identify the respective species, they proceeded utilising the experience of the same authors (Matsunaga *et al*. 1999) as the present study. Piknová *et al*. (2002) also observed that 90% out of 10 sheep and goat cheeses analysed in their laboratory contained an undeclared cow constituent. Similarly as in the present study, Kaškaval ranked among the cheeses with the positive detection of an undeclared ingredient. Di Pinto *et al*. (2004) analysed 30 mozzarella cheeses and the presence of cow's milk was found in 22 samples.

CONCLUSION

A PCR method for the determination of an undeclared quantity of cow's milk constituent in goat and sheep cheeses was validated. The 1% detection limit for the PCR reaction was determined using model cheese samples. The total of 17 samples of goat and 7 samples of sheep retail cheeses were analysed by the PCR method. It was found that 3 goat cheeses and also 1 sheep cheese contained an undeclared cow's milk constituent. One of these cheeses was of Czech origin and three of them were made in Slovakia.

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