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Species identification in dairy products by three different DNA-based techniques

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Abstract Polymerase chain reaction (PCR) with primers encoding a partial sequence of the β-casein gene was performed to detect the corresponding DNA in milk and cheese after an adapted DNA extraction procedure. In the PCR product from ovine or caprine β-casein DNA was shown to contain a specific restriction enzyme site that is not present in bovine β-casein DNA. Accordingly, after selected restriction enzyme analysis and horizontal polyacrylamide gel electrophoresis (PAGE), the undigested bovine β-casein fragment can be detected as an additional band if cow's milk is present. Appropriate experiments using unprocessed milk demonstrated that a semi-quantitative assay could be established. The detection limit was about 0.5% cow's milk in ewe's and goat's milk cheese. By use of a DNA intercalating agent the β-casein PCR products from cow or buffalo could be distinguished from those of ewe or goat as a consequence of sequence-specific retardation during agarose gel electrophoresis. Furthermore, single-stranded conformation polymorphism (SSCP) analysis was applied to detect expected species-specific conformation of the selected β-casein DNA sequences from the milk of cows, ewes, goats and buffalos milk. These techniques are compared with respect to their special use and application.

Key words DNA · Polymerase chain reaction · Single-stranded conformation polymorphism · Restriction enzymes · Cow's, ewe's, goat's and buffalo's cheese

Introduction

Within the European Union, the production of ewe's, goat's and buffalo's milk is concentrated in the southern member states and special market organisation schemes favouring those types of milk and their products do exist [1]. The importation of cheese made from ewe's, goat's or buffalo's milk, or mixtures thereof, into the European Community from certain third countries takes place under preferential arrangements, e.g. restitution of import taxes. As a consequence, adequate control methods are required to verify that no cow's milk has been incorporated into such products. Since the last review of the detection of adulteration of ewe's, goat's and buffalo's milk [2], a reference method for the detection of cow's milk based on isoelectric focusing of γ-caseins has been released by the European Community [1]. Additionally, several new methods, either immunological [3–9] or electrophoretical techniques [10, 11], have been published recently, which might be applicable to routine analysis. However, protein-based methods for species identification may fail after excessive proteolysis or heat-induced denaturation of the indicator proteins.

Genomic DNA from somatic milk cells is suggested to persist in ripened cheese and may be amplified and analysed for species discrimination. The aim of the present study was to establish quick species identification methods based on DNA extraction and amplification techniques. An adapted DNA extraction in combination with specific casein gene polymerase chain reaction (PCR) amplification/restriction was introduced. Furthermore, we compared this method with PCR/SSCP (single-stranded conformation polymorphism) and gel retardation for samples of milk from cows, ewes, goats and buffalos.

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Materials and methods

Samples. Fresh bulk milk samples from cows, goats and ewes were obtained from local farmers and served as authentic standards. Cheese samples made from ewe's, goat's or buffalo's milk containing defined amounts of raw, pasteurised or UHT-treated cow's milk or whey proteins were obtained from different European producers [12]. Heat-denatured whey proteins (Simplesse Dry 100) were from Nutrasweet, France; heat-treated milk powder and condensed milk were collected from the market.

DNA extraction. Fresh milk was centrifuged (250 ml, 400 *g*, at 4 °C for 30 min) and the resulting cell pellet was used for DNA extraction. Otherwise solid cheese or milk powder (1.5 g each) or 5 ml condensed milk was subjected to DNA extraction in the following manner: samples were homogenised on ice with 13.8 ml guanidinium isothiocyanate buffer [4 M guanidinium isothiocyanate, 50 mM Tris-HCl, 25 mM ethylenediaminetetraacetic acid (EDTA) pH 7.5] and 1.2 ml of 2-mercaptoethanol for less than 5 min, then 5 ml of chilled ethanol was added and the samples were mixed briefly. The pellets resulting from centrifugation (14,500 *g*, at 4 °C for 5 min) were dissolved in 300 µl guanidinium-HCl buffer (6 M guanidinium HCl, 25 mM EDTA pH 7.5). Then, 20 µl Silica Paramagnetic Particles (Merck, Darmstadt, Germany) was added and left for 15 min at room temperature to permit DNA adsorption to the silica particles; the tubes were mixed gently every 2 min. Using a magnetic rack to fix the particles, the buffer was removed and the particles were resuspended in 500 µl guanidinium-HCl buffer. After removing the buffer again the particles were washed twice with 70% ethanol, 50 mM Tris-HCl pH 7.2, 1 mM EDTA. Finally, the DNA-coated particles were air-dried for 20 min, resuspended in 40 µl sterile H_2O and after elution at 50 °C for 5 min the DNA-containing supernatant was stored at 4 °C until further use.

Gel filtration. DNA isolated from cheese was further purified by gel filtration in micro-spin columns (S-300 HR Columns, Pharmacia, Freiburg, Germany) to remove PCR-inhibiting substances according to the manufacturer's protocol. The DNA yield was roughly estimated by a DNA spot test: 2 µl of an ethidium bromide solution (2 µg/ml ethidium bromide in 25 mM Tris-EDTA buffer pH 8.0) and 1 µl of the DNA-containing solution or a standard dilution of calf thymus DNA (0.5–100 ng/µl) were mixed, spotted onto a transparent membrane and the resulting dots were visually compared under UV illumination.

PCR amplification. The target for PCR amplification and restriction enzyme analysis was an especially selected partial sequence of the *Bos taurus* β-casein region representing a high percentage of homology between cow (EMBL database accession no. X14711) and sheep (EMBL database accession no. X79703) genes. All primers have been chosen using the "HUSAR" online programme package in Heidelberg (http://genome.dkfz-heidelberg.de/menu/husar).

The first primer pair flanking the small 253-bp (*Bos*) or 247-bp (*Ovis*) β-casein gene fragment was used for most experiments:

forward 5′-TCC CTA AAT ATC CAG TTG AGC C-3′
reverse 5′-TCC TGG TAC AGC AGA AAG GC-3′

A second primer pair flanking the large 3622-bp β-casein gene fragment (including exons 4–7 and introns 4–6) was:

forward 5′-GAG ATT GTG GAA AGC CTT TC-3′
reverse 5′-CTT TCA GTA AAG GGC TCA AC-3′

Of the DNA extract, 1–5 µl was added to the PCR mix, comprising 200 µM each of deoxyadenosine 5′-triphosphate (dATP), deoxycytidine 5′-triphosphate (dCTP), deoxyguanosine 5′triphosphate (dGTP) and deoxythymidine 5′-triphosphate (dTTP), 500 nM of each primer, 2.5 units DNA polymerase (Expand High Fidelity, Boehringer, Mannheim, Germany), 1 × Expand HF buffer with 1.5 mM $MgCl_2$, in a final volume of 50 µl. Blank controls were included in order to detect false positives due to PCR contamination. The PCR was carried out in a thermal cycler (Biometra, Göttingen, Germany) using the following conditions: an initial denaturation step at 94 °C for 2 min followed by 35 cycles of 94 °C for 60 s and 55 °C for 60 s and a final extension step at 72 °C for 5 min. Amplified products were stored at 4 °C and separated by electrophoresis in 1% agarose gel stained with ethidium bromide. The expected sizes of the PCR products after electrophoresis were 253 bp for the bovine fragment and 247 bp for the ovine or caprine fragments.

Longer fragments (>3 kb) were amplified using adjusted cycle conditions as recommended by the manufacturer (Expand High Fidelity): 94 °C for 2 min, 10 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 3 min, 15 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 3 min including a cycle elongation of 20 s each cycle and a final extension step at 72 °C for 7 min. These samples were analysed in 0.8% agarose gels.

Table 1 Separation programme for PAGE. (A_{max} Maximum current, V_{max} maximum voltage, W_{max} maximum power)

V_{max} (V)	A_{max} (mA)	W_{max} (W)	Time (min)
200	20	10	20
375	30	20	50 for restriction (60 min for SSCP)
450	30	20	50

Restriction enzyme analysis. The amplified ovine and caprine but not the bovine β-casein sequence enclosed by the first primer pair contained an additional selected restriction site for *Ava*II and *Alu*I. Therefore, a restriction enzyme analysis was performed using 5–9 µl PCR product, 0.5–1 units *Ava*II or *Alu*I (Appligene, Heidelberg, Germany), 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 10 mM NaCl (for *Ava*II), 50 mM NaCl (for *Alu*I), 10 mM 2-mercaptoethanol in a final volume of 10 µl incubated at 37 °C for 1 h. Afterwards the fragments were separated by PAGE.

Separation of PCR products using a DNA intercalating agent. For this technique, 100 units of the DNA intercalating agent Resolver Gold (Ingenius Wiesbaden, Germany) was added to 20 ml of 2% agarose gel. Then, with 0.5 × TBE (44.5 mM Tris-HCl pH 7.5, 44.5 mM boric acid, 1 mM EDTA) as the gel running buffer, electrophoresis was carried out for 4 h at 5 V cm^{-1} and the gel was stained with ethidium bromide (0.5 µg/ml).

SSCP analysis. For SSCP analysis, 2 µl milk β-casein PCR product was mixed with 2 µl of formamide (Gibco-BRL, Gaithersburg, USA) and incubated for 7 min at 95 °C and separated by native PAGE as described previously [13].

Polyacrylamide gel electrophoresis. Electrophoresis was performed on 15% Cleangels (Pharmacia) at 15 °C with the Disc Buffer kit (Pharmacia) using the separation programme shown in Table 1.

Silver staining. The rapid and sensitive silver staining procedure described by Bassam et al. [14] was carried out to visualise the DNA pattern.

Results and discussion

DNA extraction from dairy products

Milk from healthy mammary glands contains a varying number of somatic cells (100,000–500,000/ml for the cow), representing predominantly leucocytes, less than 2% of which are thought to be epithelial mammary cells [15]. PCR, which is a DNA-based technique that can detect a small number of molecules, has been applied mainly to detect microbiological contamination in the dairy industry [16,17], but milk is also a useful substrate for PCR [18–20]. In the present study, DNA could be extracted from milk and even from processed dairy products such as cheese or heat-denatured whey proteins. This suspected genomic DNA was used as a substrate for PCR amplification of the casein-specific DNA. Of all the milk proteins investigated to date [12], the plasmin fragments of β-casein ("γ-caseins") have proved to be the most reliable indicators of species. Accordingly, a β-casein-specific gene fragment [253, 247 bp] was observed in bulk milk from cows, ewes, goats and buffalos, in spray-dried milk powder from bovine

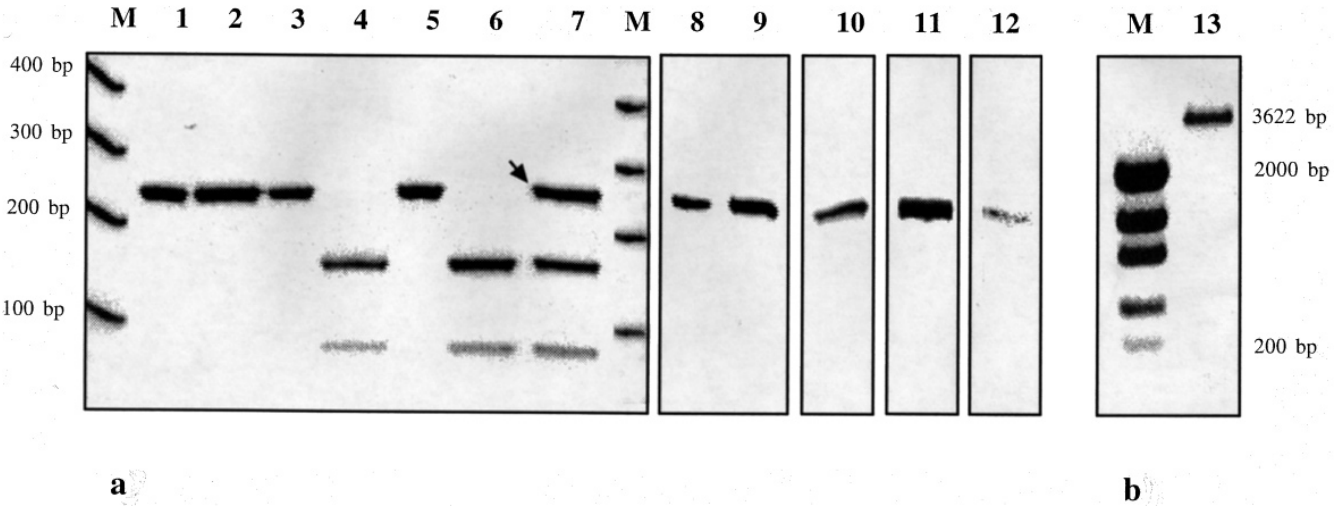

Fig. 1 a Restriction enzyme analysis of casein polymerase chain reaction (*PCR*) products using *Ava*II restriction enzyme: cow's milk undigested (*1*) and digested (*2*); ewe's milk undigested (*3*) and digested (*4*); goat's milk undigested (*5*) and digested (*6*); mixture of cow's milk and goat's milk (50/50) digested (*7*); buffalo's milk undigested (*8*) and digested (*9*); spray-dried skimmed-milk powder (*10*); heat-denatured whey proteins Simplesse Dry 100 (*11*); condensed milk (*12*). (*M* 100-bp marker (Pharmacia, Freiburg, Germany). The *arrow* indicates undigested PCR product if cow's milk was added. PAGE = 15%. **b** β-Casein PCR product (3622 bp) (*13*), mass ladder (Gibco, Gaithersburg, USA), (*M*); 0.8% Agarose gel electrophoresis

skimmed milk and even in heat-denatured whey proteins or condensed milk (Fig. 1a). An estimation of the quality of the extracted DNA was carried out by amplifying the larger 3622-bp β-casein sequence, demonstrating that fragmentation of DNA does not represent a problem. The corresponding band containing exons 4–7 and introns 4–6 could be detected in bovine milk and in some cheese made from cow's milk, suggesting that even in fermented (ripened) products relatively long DNA fragments are still present (Fig. 1b). The resulting DNA could be used for more sophisticated analysis. To prove whether DNA amplification was inhibited by cheese compounds, DNA from milk cells was added after homogenisation of cheese samples or before PCR was performed. This additional DNA could be detected after enzymatic amplification.

PCR and restriction enzyme analysis

Species discrimination based on size differentiation using the expected PCR products of 253 bp for the bovine fragment or 247 bp for the ovine/caprine fragment was not feasible using the PAGE system introduced here. Specific restriction enzyme recognition sites, exclusively located within the ovine/caprine PCR product, permitted the accurate detection of the subfamily by digestion. Two fragments of 66 and 181 bp were obtained by use of the restriction enzymes *Alu*I (which recognises AGCT) or 87 and 160 bp for *Ava*II (which recognises GGTCC). After the addition of cow's milk to goat's milk, an additional band corresponding to the undigested bovine PCR product (253 bp) was detected (Fig. 1a–lane 7). Furthermore, using the same PCR primers, it was shown that a specific product was amplified from buffalo's milk and cheese. Until now little data have been available regarding the β-casein gene of the buffalo. Our data suggest that there is a close relationship with the *Bos taurus* sequence: the PCR fragment was not recognised by *Alu*I and *Ava*II. The results of such restriction enzyme analysis are shown in Fig. 2. Randomised cheese samples adulterated with known levels of cow's milk were analysed to determine the efficiency of this method. As little as 0.5% cow's milk was detected, e.g. in the Spanish hard cheese (Fig. 2a P6-C1), demonstrating the detection limit of this system. Comparable detection limits were observed by Meyer et al. [21] in a study of meat mixtures using PCR primers for the mitochondrial cytochrome-b gene after restriction fragment length polymorphism analysis. To our knowledge this is the first attempt to measure adulteration of cheese with products from other species by DNA analysis. Nevertheless, further standardisation and validation of this technique have to be performed if it is to replace or support more laborious methods, e.g. the isolelectric focusing method described by Molina et al. [22]. As we obtained such promising results from the PCR and restriction enzyme analysis, we decided to investigate further standardised DNA techniques for their ability to detect fraudulent addition of even UHT-treated cow's milk.

As reported by Rossen et al. [23], certain food compounds, e.g. from Danish blue vein Brie and Danish Brie, may inhibit PCR. Ingredients of these special fungi-containing cheeses may interact with the DNA or the enzymatic PCR reaction; nevertheless, we obtained a PCR product from the DNA of French Roquefort cheese (Fig. 2a P8-D1). Sometimes it was not possible to identify cow's milk after restriction enzyme analysis (Fig. 2a, Feta P3-B1, Kefalotyri P9-D1 and French soft cheese P1-B3). However, in these cases the low efficiency of the PCR did not yield an amount of DNA that was great enough to be used for restriction enzyme analysis, and use of more than 40 PCR cycles results in the formation of amplification artefacts.

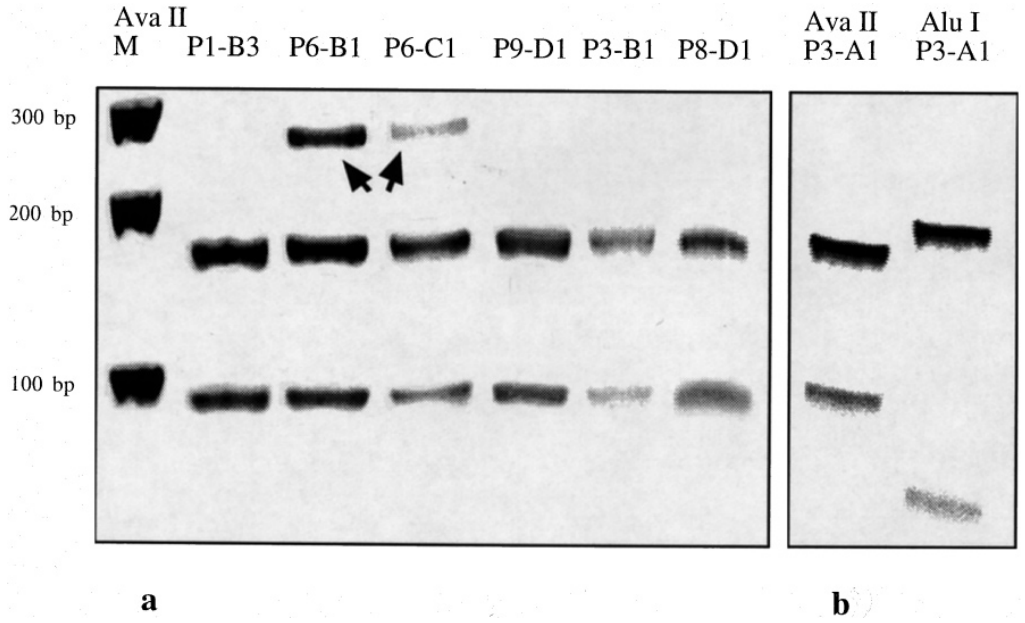

Fig. 2a, b Restriction enzyme analysis of cheese samples. **a** Using *Ava*II: French soft cheese (*P1-B3*, 65/35 ewe/goat; 0.5% cow's milk); Spanish hard cheese (*P6-B1* and *P6-C1*, 50/50 ewe/goat; 2% and 0.5% cow's milk, respectively); Greek Kefalotyri (*P9-D1*, 75/25 ewe/goat; 0.5% cow's milk); Greek Feta (*P3-B1*, 50/50 ewe/goat; 1% cow's milk); French Roquefort (*P8-D1*, 100% ewe, 0% cow's milk). The *arrow* indicates undigested PCR product (257 bp) only present if cow's milk was added before cheese production. (*M* 100-bp marker, Pharmacia) PAGE = 15%. **b** Greek Feta (*P3-A1*, 75/25 ewe/goat; 0% cow's milk), using *Ava*II and *Alu*I restriction enzymes. PAGE = 15%

PCR and agarose gel electrophoresis using an intercalating agent

The small size differences between the bovine (253 bp) and the ovine/caprine (247 bp) β-casein gene fragments could not be distinguished by PAGE, as described above. Nevertheless, β-casein PCR products of similar size but different sequences migrated at different velocities when the intercalating agent (Resolver Gold) was used, thus enabling differentiation between cow and buffalo and between sheep and goat (Fig. 3). As is demonstrated, an obvious retardation during gel migration is observed between these two groups. This technique allows the qualitative distinction between pure species but fails to analyse distinct quantities of adulteration: the two co-migrating bands are not really distinct and adulterations below 50% are barely detectable (Fig. 3). These results do not suggest that such an intercalating technique could be usefully applied to this type of analysis.

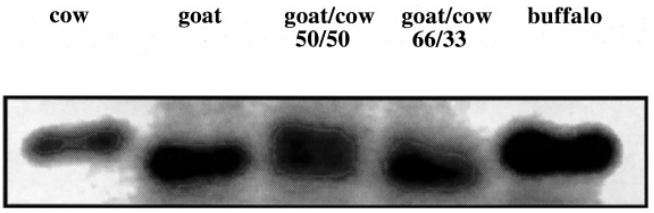

Fig. 3 Separation of PCR products using a DNA intercalating agent during agarose gel electrophoresis (2%). Analysis of cow's, goat's or buffalo's milk samples and mixtures of goat's and cow's milk (50/50 and 66/33, respectively)

PCR and SSCP

A third technique based on differences in the conformation of DNA, which depends directly on single sequence differences, was than applied to species differentiation. PCR/SSCP can detect single, even unknown, point mutations. This technique revealed a species-specific band pattern for milk from cows, buffalos, ewes and goats (Fig. 4). A mixture of goat's and cow's milk (50/50) could be clearly detected (Fig. 4), but this method does not seem to be sensitive enough or adequate for the detection of low levels of adulteration with cow's milk when compared with PCR/restriction enzyme analysis. Sample dilution during single-strand analysis and multiple band-splitting are possible reasons for the lower sensitivity. Nevertheless, PCR/SSCP is even more rapid than the other techniques discussed and

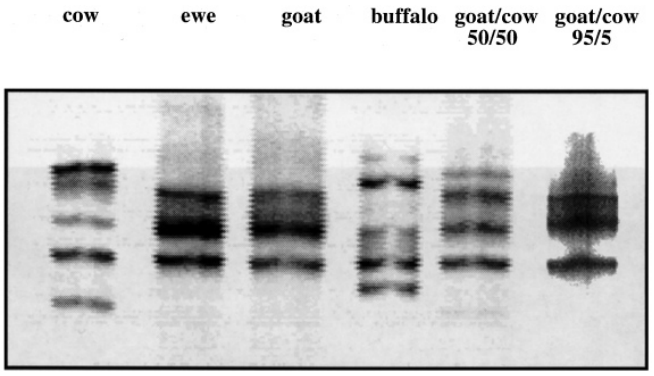

Fig. 4 Single-stranded conformation polymorphism (*SSCP*) analysis of milk. Cow's, ewe's, goat's or buffalo's milk samples and mixtures of goat's and cow's milk (50/50 and 95/5, respectively). PAGE = 15%

can be used to distinguish cow's or buffalo's milk from ewe's milk or goat's and cow's milk from buffalo's milk. Furthermore, it allows detection of unknown, single mutations and subsequently the predicted DNA polymorphism of the casein gene fragment may be used to distinguish between different breeds within a species.

In conclusion, the casein PCR amplification method described is applicable to the analysis of cheese made from mixtures of ewe's and goat's milk. The PCR-based DNA analysis is fast and sensitive and even severe heat treatment had no influence on its ability to detect bovine dairy products. The quality of the purified DNA is still an unsolved problem in distinct cases and we suggest that cheese ingredients may lead to poor PCR amplification. Future work should focus on increasing the quality of the DNA after extraction and on obtaining a more quantitative validation of the PCR methods. Our first attempts suggest that remnant DNA may be used for a quick analysis of the prior adulteration of processed food. Three different DNA analysis systems have been introduced, each representing unique features of application. In our hands, only PCR amplification combined with endonuclease treatment of the resulting PCR product was effective for semi-quantitative assays. In general, DNA analysis using PCR followed by restriction enzyme analysis represents a powerful tool for species discrimination, at a time when it is becoming more important to prove the origin of the product within the dairy industry.

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