Application of polymerase chain reaction for detection of goats' milk adulteration by milk of cow

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Numerous methods based on DNA analysis have been employed in the food industry to monitor adulterations of food products of animal origin. Among them the most frequently used are: polymerase chain reaction (PCR) amplification of a marker gene fragment(s) with universal primers, or amplification of DNA with species-specific primers. PCR-products of different origin can be discriminated by size, restriction fragment length polymorphism (RFLP) or single stranded conformational polymorphism (SSCP) analysis. These methods have been used for identification, and differentiation between, the animal origins of raw or heat-treated meat and meat products (Chikuni *et al*. 1994; Meyer *et al*. 1994, 1995; Zehner *et al*. 1998; Behrens *et al*. 1999; Guoli *et al*. 1999; Hopwood *et al*. 1999; Matsunaga *et al*. 1999; Wolf *et al*. 1999). These approaches are also applicable to the analysis of dairy products. However, adulterations of goats' milk and its products are traditionally tested by immunological and/or electrophoretic methods (Amigo *et al*. 1992; Levieux & Venien, 1994; Mimmo & Pagani, 1998). So far, only a few DNA-based techniques designed to detect the presence of bovine DNA in goats' milk have been described (Plath *et al*. 1997; Branciari *et al*. 2000). This paper presents a one-step PCR procedure for detection of adulteration of goats' milk with cows' milk. The method, employing bovine-specific primers for amplification of a 274 bp fragment of cytochrome b DNA, seems to be simple, fast, specific and sensitive.

materials and methods

Goats' milk samples were obtained from local farmers, and samples of cows' milk, UHT and pasteurized, were purchased from different commercial sources.

DNA extraction

Individual milk samples (0·4 ml) were mixed with 200 μl 200 mM-Tris-HCl, pH 8·0, 100 mM-EDTA, 10 g SDS/l and 0·6 mg of protease from *Streptomyces griseus*

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(Sigma, St Louis, MO 63178, USA). The mixture was incubated for 1 h at 55 °C and then extracted twice with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, by vol.) and once with an equal volume of chloroform. After addition of 30 μl 7·5 M-ammonium acetate, DNA was precipitated with two volumes of ethanol and pelleted by centrifugation at 12000 ***g*** for 2 min (Sambrook *et al*. 1989). The pellet was dried and re-dissolved in TE buffer (10 mM-Tris-HCl, pH 8·0, 1 mM-EDTA). The concentration of DNA was measured by u.v.-absorption spectroscopy at 260 nm.

PCR reaction

The reaction mixture for PCR contained: 10 mM-Tris-HCl, pH 8·8, 1·5 mM-$MgCl_2$, 50 mM-KCl, 1 g Triton X-100/l, 0·2 mM-dNTPs mix (Sigma), 40 pmole of each primer (Bionovo, Legnica 59220, Poland), 2 units Taq polymerase (Finnzymes, Espoo FIN 02201, Finland) and DNA template added in a volume not exceeding 3 μl. Bovine cytochrome b 274-bp DNA fragment was amplified with forward primer: 5′ GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA 3′, and reverse primer: 5′ CTAGAAAAGTGTAAGACCCGTAATATAAG 3′ (Matsunaga *et al*. 1999). Reaction was conducted in a total volume of 50 μl. After the initial denaturation step at 94 °C for 1 min, 2 units of Taq polymerase were added and 32 cycles of amplification were performed in a Uno Thermoblok cycler (Biometra, Gottingen D-37079, Germany) using the following parameters: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. PCR products (0·2 of the total sample) were separated at 100 V for 30 min in a 20 g/l agarose gel containing 0·6 μg ethidium bromide/ml in TAE buffer (40 mM-Tris-acetate, pH 8·0, 1 mM-EDTA).

RESULTS AND DISCUSSION

Varying volumes of cows' milk, up to 50 % of total sample, were added to goats' milk. From such samples DNA (approximately 10 μg/ml) was isolated and subjected to PCR with bovine-specific primers for the fragment of cytochrome b gene. After 32 cycles of amplification, an intensive band of $\sim$ 274 bp was found only in samples containing cows' milk (Fig. 1).

To our knowledge only a few reports exist on the use of PCR that help distinguish cows' and goats' milk and products (Plath *et al*. 1997; Branciari *et al*. 2000). In the method described by Plath *et al*. (1997) amplified β-casein gene fragments common to goat and cow were discriminated by RFLP analysis. Recently, Branciari *et al*. (2000) have described a similar procedure for identification of water buffalo, bovine, ovine and caprine milk in cheese, based on amplification of a 359 bp fragment of the cytochrome b gene.

In contrast to the methods discussed above, our approach, based on the use of a bovine-specific primer set, represents a single-step technique without the need for the subsequent restriction fragment analysis. The primer pair used is one of the sets of primers described by Matsunaga *et al*. (1999), which were designed originally to identify DNA isolated from meat and meat products of six different species. The sequence of the forward primer was based on a highly conserved region of the mitochondrial cytochrome b gene, common to all amplified DNA, and the reverse primers were constructed using species-specific DNA sequences of the target gene. These primers were used for simultaneous identification of meats of different origin in the multiplex PCR method, as species-specific primer sets gave DNA fragments of different sizes. As we have found, with such primers the number of PCR cycles for amplification of DNA isolated from goats' milk adulterated with cows' milk was

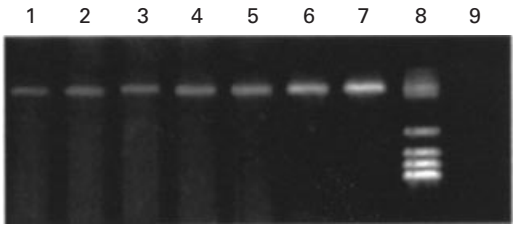

Fig. 1. Agarose (20 g/l) gel electrophoresis of polymerase chain reaction products obtained with bovine-specific primers for a 274 bp fragment of the bovine cytochrome b gene. Lanes 1–7 represent mixtures of goats' milk containing 0·1, 0·5, 1, 5, 10, 20, and 50% cow's milk respectively; lane 9: sample of goats milk only; lane 8: molecular markers, ϕX174 RF DNA *Hae*III digest.

critical. After 35 cycles, as used by Matsunaga *et al.* (1999), DNA isolated from caprine milk, with bovine-specific primers produces a non-specific band migrating similarly to the DNA fragment obtained from bovine DNA. However, this non-specific band was not seen in samples of pure goats' milk when the number of PCR cycles was decreased to 32. Thus, the sensitivity of the method for detecting bovine DNA was strongly influenced by the number of cycles used.

DNA isolated from milk is of somatic cell origin. For this reason, in the initial experiments, the somatic cells were pelleted prior to DNA extraction. However, we subsequently found that direct treatment of uncentrifuged milk samples with proteolytic enzymes, followed by extraction with an equal volume of phenol–chloroform–isoamyl alcohol mixture, does not affect the sensitivity of the method. As milk samples from healthy cows vary in the number of somatic cells contained (100000–500000/ml), the DNA content also differs considerably. To determine the efficiency of the method, all samples of goats' milk were spiked with cow UHT milk, as this milk, including the number of somatic cells – which according to Polish regulations can not exceed 400000/ml, is the subject of strict control (Polish regulation No. PN-A-86002, 1995 and No. PN-A-86003, 1996). The addition of as little as 1 ml/l of cows' UHT milk to goats' milk was detected by this method. This was in agreement with the results of others, who have used PCR for tracing the origins of various food items to their respective source animals (Meyer *et al.* 1994; Plath *et al.* 1997).

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