Determination of the animal origin of raw food by species-specific PCR

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Summary. Specific PCR, amplifying a fragment of the mitochondrially encoded *cytochrome b* gene, are developed for discrimination of chicken, turkey, pig, cow and sheep. These PCR tests could be applied for detection and discrimination of animal food ingredients. For origin identification of milk, the bovine PCR is applied on DNA extracted with the DNeasy™ Tissue kit. A PCR of 30 cycles reached a sensitivity of 720 somatic cells in the PCR reaction corresponding to $2{\cdot}9 \times 10^4$ cells per ml milk. A PCR of 40 cycles could detect 0·72 to 0·144 somatic cells in the PCR reaction corresponding to 29 to 2·9 cells per ml milk.

Keywords: PCR *cytochrome b*, milk, animal identification.

The quality of food products, including food authenticity, is an important concern for modern consumers, resulting in an increasing pressure on governmental policy and different levels of the food production chain. For example, food inspectors check food products for accurate labelling for economic (e.g. selling cheaper meats), religious or health reasons. For the detection of animal food ingredients and their origin identification, adequate analytical tools are needed. PCR offers the possibility to detect animal food ingredients and to identify them as being derived from a certain animal species.

For species identification, a specific PCR can be developed, targeting a DNA segment with sufficient species-to-species variation. Mitochondrially encoded genes as the ATPase subunit 8 and subunit 6 (Tartaglia *et al*. 1998) and the *cytochrome b* genes are studied for this purpose. It has been shown that the *cytochrome b* sequences differ by at least a few nucleotides even in very closely related species (Martin & Palumbi, 1993). A standard set of primers, directed towards conserved regions of the *cytochrome b* gene, allows the amplification from more than 100 animal species (Kocher *et al*. 1989). DNA sequence analysis of the amplified fragment and subsequent phylogenetic analysis using a database can reveal the species identity (Bartlett & Davidson, 1992). In this article the *cytochrome b* system is used to develop species-specific primers which allow discrimination between important domestic animals. This species-specific PCR is subsequently applied for the origin identification of milk.

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Bovine milk contains a number of somatic cells varying from 10^4 to 10^7 per ml and is therefore an easy source for animal DNA. The number of somatic cells in bovine milk is greatly dependent on the clinical status of the cow. In this article, DNA was extracted by using the DNeasy™ Tissue kit (Qiagen, Valencia, USA). The sensitivity of the DNA extraction and the bovine discriminative PCR has been determined by diluting somatic cells and extracted DNA.

In this paper, we describe a novel PCR method, which is useful for ascertaining the species origin of meat samples. Moreover, we report the optimisation of a method for isolating genomic DNA from milk samples and demonstrate that this DNA can be PCR amplified.

MATERIALS AND METHODS

Samples and DNA extraction

Different meat portions were obtained from the retail shop. DNA was extracted from an internally located 25 mg of beef, pork, chicken and turkey meat using the DNeasy Protocol for Animal Tissue provided with the DNeasy™ Tissue kit (Qiagen) with a concentration of 66 ng/μl, 191 ng/μl, 100 ng/μl and 147 ng/μl, respectively.

Partial cloning and sequencing of the cytochrome b gene from different mammalian and avian species

Part of the *cytochrome b* sequence was amplified using for chicken and turkey cyt bL and cyt bH (Bartlett & Davidson, 1992) which are a modification of the primers L14841 and H15149, published by Kocher et al. (1989) and for cow, pig, sheep, horse and goat FINS F and FINS R (Fig. 1). The PCR product was purified using the High Pure PCR Product Purification kit (Boehringer Mannheim, Mannheim, Germany). The purified PCR fragment was cloned in a pMOS*Blue* blunt-ended vector (Amersham, Buckinghamshire, UK) as described by the manufacturer. After overnight ligation at 15 °C, the recombinant plasmid was transformed in competent *Escherichia coli* DH5α (Life Technologies Inc., Gaithersburg, MD, USA) and the cell suspension was plated on Luria Broth (LB) agar (Life Technologies) containing carbenicilline (150 μg/ml), 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal), and isopropyl β-D-thiogalactopyranoside (IPTG) by a standard protocol (Sambrook *et al.* 1989). White colonies were subjected to clone analysis as described by Birnboim and Doly (1979). Recombinant plasmid DNA was prepared by the Qiagen Plasmid Mini Kit (Qiagen) and the inserts were sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), and vector-directed primers T7 and U19 (the 19-mer primer as indicated by the manufacturer, Amersham). The results were analysed on a 373A automated DNA sequencer (Applied Biosystems). Sequence alignment was made with the GeneCompar software package (version 2.0, Applied Maths, Kortrijk, Belgium).

PCR amplification

PCR was performed in a final volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8·3), 1·5 mM $MgCl_2$, 200 μM each dNTP, 1·5 U AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT, USA), 50 pmol each primer and 1 μl DNA as obtained by using the DNA extraction protocols, described above. The mixture was subjected to 30 or 40 cycles of amplification in a thermal cycler (Cetus 9600; Perkin Elmer). The first cycle was preceded by denaturation for 1 min at 95 °C. Each cycle consisted of denaturation for 15 s at 95 °C, annealing for 30 s at the indicated temperature

forward primer (F, 3'→5')	reverse primer (R, 3'→5')	references*
AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA	L14841-H15149
CCATCCAACATCTCAGCATGATGAAA	CCCCTCA AATGATATTTGTCCTA	cyt bL-cytbH
CTTCCCACACCATTTAACATTTCTATATGATGAAA	TACTGTAGTGCCTCAGAAAGATATCTGGCCTTA	hsac2087
CTCCCCGCTCCCTCAAGCCTATCATCATGATGAAA	GACGGTAGCTCCTCAAAAAGATATTTGTCCTCA	phcyb
CTCCCCGCCCCTTCAAGCCTCTCATCATGATGAAA	GACGGTGGCTCCTCAGAATGATATTTGTCCTCA	cdcyb
A(A/G)C(A/C)TCTC(AAC/TTT)ATGATGAAA	CCTCA(G/A)AA(T/A)GATAT(T/C)TG(T/G)CCT	FINS F-FINS R

Fig. 1. Alignment of primers used for amplification of the *cytochrome b* fragment. References*: L14841–H15149, Kocher *et al*. 1989 (human sequence); cyt bL–cyt bH, Bartlett & Davidson, 1992 (human sequence) annealing temperature 53 °C; hsac2087 (F: 66077–66043), EMBL accession of human *cytochrome b* sequence; phcyb (F: 61–95; R: 435–403), EMBL accession of *Platyrrhinus helleri cytochrome b* sequence; cdcyb (F: 61–95; R: 435–403), EMBL accession of *Chiroderma doriae cytochrome b* sequence; FINS F-FINS R, this work, annealing temperature 50 °C.

(Fig. 1, Table 1), and elongation for 30 s at 72 °C. The last cycle was followed by a final elongation for 8 min at 72 °C. The PCR products were analysed on a 1·5 % (w/v) Seakem ME agarose gel (FMC Bioproducts, Rockland, ME, USA).

We confirmed that our primers were species-specific in each one of the five species studied. With this purpose, we serially diluted variable amounts of genomic DNA from each one of the species with a mixture of genomic DNA pooled from the remaining four species. For instance, 50 ng pooled DNA (pig, sheep, chicken and turkey) were added to 50 ng, 5 ng, 0·5 ng, 0·05 ng and 0·005 ng of bovine DNA and used as a template for PCR amplification.

Determination of PCR sensitivity in bovine raw milk

Bovine milk, applied as standard in the Belgium ring trial for somatic cell count determination, was used for isolating genomic DNA. The milk contained $2{\cdot}9 \times 10^5$ somatic cells per ml as was determined with the Fossomatic 360 (Foss Electric, Hillerød, Denmark; Van Crombrugge, 1989; Laevens *et al*. 1996). Bronopol (0·025 %, Knoll Pharmaceuticals, Mount Olive, NJ, USA) and sodium azide (0·0075 %) were added as preservatives, and the milk was kept at 4 °C. Dilutions of this milk were made in 0·9 % NaCl and in reconstituted skimmed milk (10 % skimmed milk powder in H_2O) which was first filtered through a 5-μm AE98 filter (Schleicher & Schuell, Dassel, Germany) and sterilised for 30 min at 121 °C. It was confirmed by PCR that this combination of filtration and sterilisation for a prolonged period was necessary to destroy sufficiently traces of DNA from somatic cells. DNA was extracted using the DNeasy™ Tissue kit (Qiagen.) in a total volume of 200 μl elution buffer. We used as a template 10 μl and 5 μl of the DNA preparation depending on the DNA source (NaCl diluted milk or reconstituted skimmed milk, respectively). Fat was extracted from raw milk as described by Herman *et al*. (1995) except that ethanol was replaced by *n*-propanol. To 1 ml of raw milk, 200 μl NH_3, 200 μl *n*-propanol, 300 μl diethyl ether, 150 μl petroleum ether, and 0·07 % sodium dodecyl sulphate (SDS) were added and, after centrifugation at 7,500 ***g*** for 15 min, the upper layer was removed.

RESULTS

Development of species-specific PCR for domestic animals

The primers cyt bL and cyt bH (Bartlett & Davidson, 1992) resulted in a light but sufficient amplification of the mitochondrial *cytochrome b* fragment from chicken and

Table 1. *Primer sequences and annealing temperatures for each one of the species-specific PCRs*

	Forward primer (F)		Reverse primer (R)		
Animal†	Sequence (5′–3′)	Position§	Sequence (5′–3′)	Position	AT‡
Chicken	CGGTGGCTATGAGTGTGAGG	F : 311–292	AGCAGTCTGCCTCATG	R : 43–58	67 °C
Turkey	TATGAGGGTGAGAAGTAA	F : 304–287	TAGCAGTATGCCTCATCACT	R : 42–61	63 °C
Pig	GTTGCTATAACGGTAAAT	F : 304–287	TAGGCATCTGCCTAATCTTG	R : 37–56	60 °C
Cow	GGACGTATCCTATAAAT	F : 323–307	GGAATCTGCCTAATCCTA	R : 38–55	57 °C
Sheep	ATGCTGTGGCTATTGTC	F : 305–289	CCTAGGCATTTGCTTAATTTTA	R : 31–52	60 °C

†Chicken, *Gallus gallus*; turkey, *Meleagris gallapavo*; pig, *Sus scrofa*; cow, *Bos taurus*; sheep, *Ovis aries*.
‡AT: annealing temperature.
§The positions correlate to numbers in Fig. 2.

turkey. No amplification was obtained from the mammals tested. Alignment with primers L14841 and H15149 (Kocher *et al*. 1989) revealed some important differences: cyt bL and cyt bH were 9 nucleotides shorter at the 5′ end and two nucleotides were deleted in cyt bH (Fig. 1). Alignment with mammalian sequences available in the EMBL database showed more deviations, especially in the reverse primer. Based on this alignment, the primers FINS F and FINS R were designed (Fig. 1) for amplification of the *cytochrome b* fragment in pig, cow and sheep. The nucleotide sequence at positions 350 to 361 was aligned for the domestic animals involved in this study (Fig. 2). Based on this alignment PCR primers were designed which allow discrimination between different animal species (Table 1).

We tested our species-specific primer pairs amplifying *cytochrome b* from meat-extracted DNA in each one of the five studied species (cattle, pig, sheep, chicken, turkey). As expected, we never observed any cross-amplification event between different species. DNA (50 ng) of the five domestic animals was mixed and a specific reaction was obtained with each of the individual species-specific primers (Fig. 3). With the pig-specific and the cow-specific primers, 0·5 ng DNA could specifically be detected in a mixture of 50 ng of the other meat samples. The chicken, turkey and sheep primers reached a detection sensitivity of 0·05 ng of target DNA.

Detection of bovine DNA in milk by PCR

DNA was extracted from milk using the DNeasy™ Tissue kit (Qiagen). The milk fat did not influence the extraction efficiency as was tested by comparing the bovine PCR sensitivity before and after chemical extraction of the milk fat. This indicates that the fat extraction procedure had no effect on the lysis of the somatic cells as had been observed for bacterial cells (Herman *et al*. 1995).

The sensitivity of the PCR on bovine milk was determined by diluting milk with a somatic cell count of $2{\cdot}9 \times 10^5$ per ml in 0·9 % NaCl as well as in reconstituted skimmed milk. The DNA isolated from somatic cells was detected with the PCR, specific for *Bos taurus* and the sensitivity of 30 and 40 PCR cycles was compared (Table 2, Fig. 4). With a PCR of 30 cycles, 720 somatic cells in the PCR reaction could be detected, which corresponds to a somatic cell count of $2{\cdot}9 \times 10^4$ per ml milk. The same sensitivity was obtained with somatic cells diluted in 0·9 % NaCl and in skimmed, sterilised milk. With a PCR of 40 cycles, 0·144 and 0·72 somatic cells in the PCR reaction could be detected in 0·9 % NaCl and in skimmed sterilised milk, respectively. This sensitivity corresponds with the presence of 2·9–29 somatic cells per ml milk.

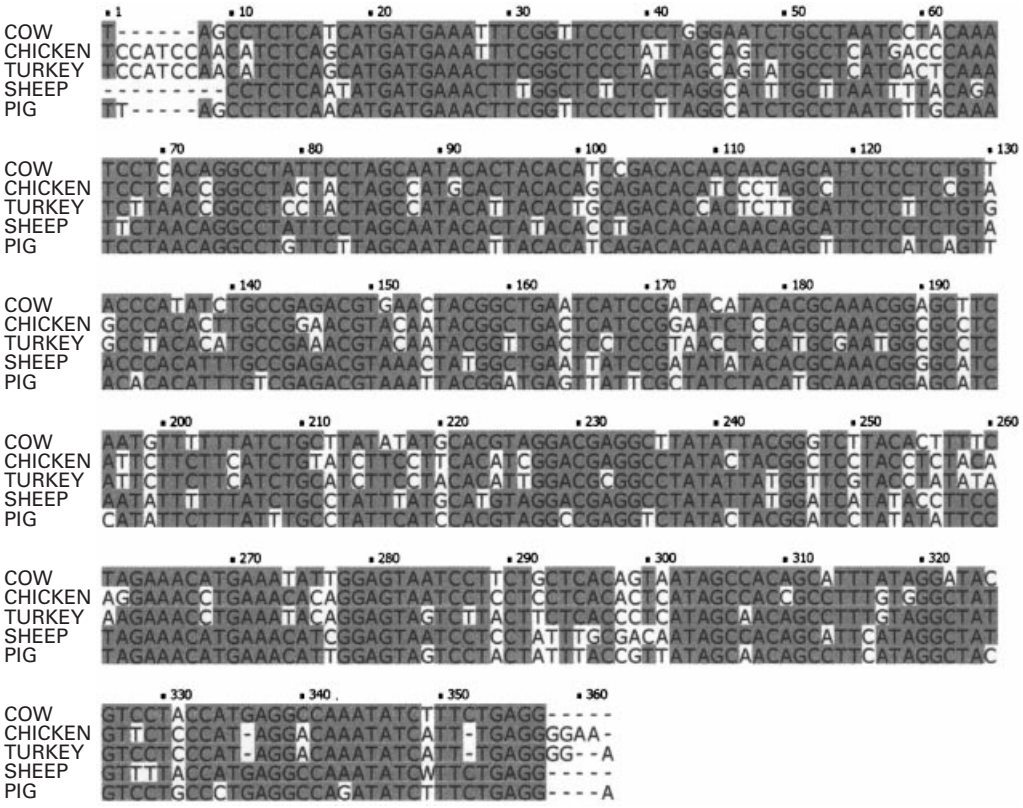

Fig. 2. Sequence alignment of a mitochondrial *cytochrome b* fragment from different domestic species. The sequences were submitted to the EMBL database: cow, *Bos taurus*: AJ401081; chicken, *Gallus gallus*: AF401080; turkey, *Meleagris gallopavo*: AF401084; sheep, *Ovis aries*: AJ401083; pig, *Sus scrofa*: AF401082.

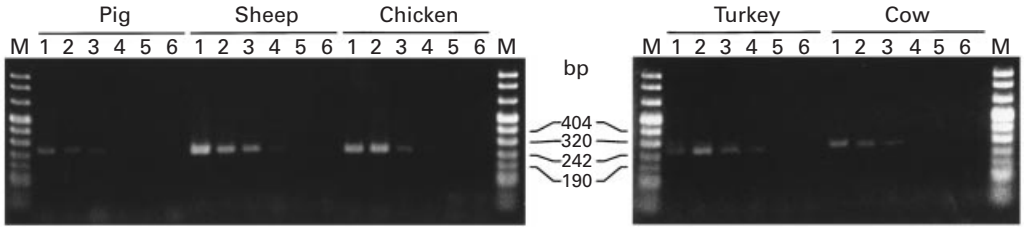

Fig. 3. Sensitivity of the species-specific PCR method in a mixture of aspecific DNA. The sensitivity of the species-specific PCR was evaluated by mixing variable amounts of the target DNA of one species (SP) with 50 ng of genomic DNA pooled from the remaining four species (control). Lane 1, 50 ng SP + 50 ng control DNAs; Lane 2, 5 ng SP + 50 ng control DNAs; Lane 3, 0·5 ng SP + 50 ng control DNAs; Lane 4, 0·05 ng SP + 50 ng control DNAs; Lane 5, 0·005 ng SP + 50 ng control DNAs; Lane 6, PCR negative control; M, molecular weight marker set VIII (Boehringer Mannheim).

DISCUSSION

In this paper, species-specific PCR are developed for species discrimination of important domestic animals. For this purpose, the mitochondrially encoded *cytochrome b* gene was chosen as a target. For obtaining sequence data, the basic

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Table 2. *PCR sensitivity for the detection of bovine DNA on milk*

Number of somatic cells in the PCR reaction†	Bovine PCR detection‡			
	Dilutions in 0·9% NaCl		Dilutions in skimmed milk	
	30 cycles	40 cycles	30 cycles	40 cycles
1440	+	+	+	+
720	+	+	+	+
144	−	+	−	+
72	−	+	−	+
14·4	−	+	−	+
7·2	−	+	−	+
0·72	−	+	−	+
0·144	−	+	−	−
0·072	−	−	−	−

†DNA was extracted from somatic cells diluted in milk and an appropriate fraction as described in the text was added to the PCR mix.
‡ + amplification of the bovine PCR product; − no amplification of the bovine PCR product.

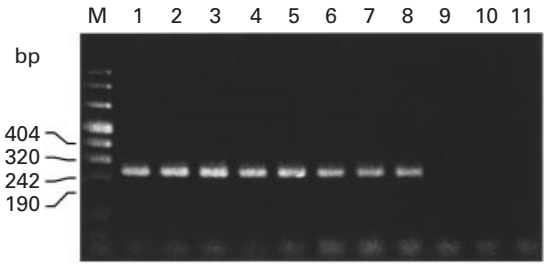

Fig. 4. Sensitivity of PCR in detecting bovine DNA in milk. DNA was extracted from diluted milk containing a varying number of somatic cells and an appropriate fraction as described in the text was added to the PCR mix. Lane 1, 1440 somatic cells; Lane 2, 720 somatic cells; Lane 3, 144 somatic cells; Lane 4, 72 somatic cells; Lane 5, 14·4 somatic cells; Lane 6, 7·2 somatic cells; Lane 8, 0·72 somatic cells; Lane 9, 0·144 somatic cells; Lane 10, 0·072 somatic cells, Lane 11, PCR negative control; M, molecular weight marker set VIII (Boehringer Mannheim).

strategy proposed by Bartlett & Davidson (1992) was followed based on primers published by Kocher *et al*. (1989). For efficient amplification of the *cytochrome b* genes of different mammals, however, new adopted primers were designed.

Based on an alignment of the *cytochrome b* gene, species-specific PCR can be developed for important domestic animals. These species-specific PCR can be applied for confirming food authenticity because a specific sequence can be detected very sensitively in a pool of sequences of different origin. This strategy has been followed by Tartaglia et al. (1998) who developed a specific PCR for the detection of bovine DNA in animal feed.

The species-specific PCR could be applied for confirming the origin of milk and milk products. This is not only important for detection of fraudulent manipulations (e.g. mixing of cheaper bovine milk in cheeses derived from sheep or goat milk) but also for prevention of food-borne allergic reactions derived from milk proteins. A possible application could be the detection of traces of milk in certain milk-free chocolates. As an alternative to detection by PCR, either iso-electric focusing (EC Regulation no. 1081/96; Molina *et al*. 1995), enzyme-linked immunosorbent assays (Haza *et al*. 1999) or capillary electrophoresis (Cartoni *et al*. 1998) can be applied for

these purposes with the possible advantage of obtaining (semi) quantitative results. In contrast, PCR-based techniques offer a high detection sensitivity and specificity. PCR can also be applied in highly processed food products with extensive protein denaturation.

A simple DNA extraction method was used in combination with PCR which was shown to be highly efficient: a PCR of 30 cycles could detect 720 somatic cells per PCR reaction, a PCR of 40 cycles reached a sensitivity of 0·72 to 0·144 cells. Lipkin et al. (1993) have reported a DNA extraction method from raw milk, based on a phenol:chloroform-*iso*-amyl alcohol extraction, with a sensitivity varying between 100 cells to 2500 cells per PCR reaction of 40 cycles. Less variable results were obtained by using a Chelex resin in the DNA purification leading to a sensitivity of 100 cells to 150 cells per PCR reaction of 45–65 cycles (Amills *et al.* 1997). With their method, no amplification product has been obtained from any sample at 30 cycles. The higher sensitivity obtained using our method is probably due to the higher copy number of mitochondrial genes, used as target in our method, compared to the chromosomal genes used by others. This idea is supported by the fact that a 10–100-times increase in PCR sensitivity was observed with the mitochondrial target relative to two different chromosomal targets (data not shown). We used a commercial kit to avoid the use of hazardous chemicals and for convenience.

Bovine milk contains 10^4–10^7 somatic cells per ml. The European legislation sets an upper limit of 4×10^5 per ml. With a detection sensitivity of $2{\cdot}9 \times 10^4$ somatic cells per ml with a PCR of 30 cycles and of 2·9–29 cells per ml with a PCR of 40 cycles, the method, described here, reaches sufficient sensitivity for authenticity tests on milk and milk products.

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