Animal species identification and quantification in meat and meat products by means of traditional and real-time PCR

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Abstract

The objective of the presented investigations was to contribute to the optimisation of DNA based methods for the identification of various animal species (poultry and ruminants) that are of importance in human nutrition and furthermore to find possibilities for quantification of animal species in meat products. An essential prerequisite for animal species identification and especially quantification is the reproducible isolation of intact DNA to be applied in traditional and real-time PCR. Among a variety of commercially available DNA isolation kits an optimised classical CTAB protocol proved to be most suitable. Using sequences of the mitochondrial genome the limit of detection is 100 to 1000 fold lower than in case of nuclear single-copy genes. However, only single-copy genes of the nuclear genome are appropriate for species quantification by real-time PCR. A semi quantitative PCR system for the determination of goat tissue in meat products was developed based on the nuclear single-copy gene *beta-Casein*. Furthermore it could be shown that it is possible to differentiate clearly between the most common poultry species used in Europe as well as ruminants like sheep, goat, cattle, bison and buffalo based on mitochondrial primer systems.

Introduction

Food production chain is increasingly becoming complex. Nevertheless, consumer should be offered a wide range of safe and high quality products. Every link in the chain must be as strong as the others if health and well-being of the consumers is to be adequately protected. This includes developing systems to link products to their source, identify components, counteract fraud and insure that proper processing has taken place. Fraud detection includes the ability to authenticate with respect to food labelling as well as compliance with ingredient composition. Aim of the presented study was the authentication of the most common domestic poultry species (chicken, turkey, duck, goose, pheasant, quail and guinea fowl) in Europe (Stirtzel et al., 2007) as well as ruminants like sheep, goat, cattle, bison and buffalo in meat and meat products and thereby to provide a method to detect adulteration. Within this DNA based investigations mitochondrial *cytochrome b* gene sequences were applied for interspecies comparison and nuclear single-copy gene fragments were used to develop semi quantitative PCR systems for the determination of goat tissue in meat products (Altmann et al., 2004). For the adaptation and development of reliable qualitative as well as quantitative polymerase chain reaction (PCR) systems the reproducible isolation of intact DNA is the most essential prerequisite for a successful molecular biological analysis. Therefore a classical CTAB protocol was optimised (Binke et al., 2003).

Material and methods

I) DNA isolation

For the extraction of nucleic acid 25 mg to 100 mg muscle meat of various animal species or 50 mg of various meat products were used. The isolation was carried out by means of an optimized CTAB method (Binke et al., 2003). Different batches of reference sausages were produced containing 50% muscle meat with various amounts of the different animal species, 25% sunflower oil, 23% ice, 2% salt, phosphate, ascorbate and nitrite. Each batch was heat treated at F_C-values of <0.9, 0.9, 3.4, 12.3 and 32. Classical PCR and real-time PCR were performed applying a specific PCR protocol (Binke et al., 2003).

II) Differentiation of animal species

1) Poultry

Some species specific primer systems were adapted from literature: chicken (*gallus gallus*), goose (*anser cygnoides*) and duck (*cairina moschata*). The mitochondrial (mtDNA) sequences coding for *cytochrome b* for the species turkey (*meleagris gallopavo*), pheasant (*phasianus colchicus*), quail (*coturnix coturnix*) and guinea fowl (*numida meleagris*) were searched for in various genomic databases such as genbank (National Centre for Biotechnology Information, NCBI) and EMBL (European Molecular Biology Laboratory) using the Entrez Global Query Cross-Database Search system of NCBI and the Sequence Retrieval System (SRS) of EMBL. Sequence alignment using CLUSTAL (multiple Sequence Alignment) as

well as the cross checks against various nucleotide databases (Basic Logical Alignment Search Tool, BLASTN) were done using the HUSAR Sequence analysis package (Heidelberg UNIX Sequence Analysis Package W2H 4.1) of the German Cancer Research Centre. DNA was extracted as described by Binke et al. (2003).

Meat samples and processed meat products containing the above mentioned species were purchased from local markets. PCR was carried out using the Thermocycler GeneAmp PCR System 9600 (Perkin-Elmer GmbH, Überlingen, Germany). The master mix was composed as follows: 16,3 µL PCR-water, 2,5 µL PCR-buffer (tenfold concentrated, Qiagen, Hilden, Germany), 1,0 µL deoxynucleotide triphosphate (dNTP) -mixture (10 mM per dNTP, Qbiogene, Heidelberg, Germany), 1,0 µL per primer (forward and reverse, c = 10 µM, Operon, Köln, Germany, 1,0 µL $MgCl_2$ (25 mM, Qiagen, Hilden, Germany) and 0,2 µL HotStarTaq™ DNA-polymerase (5 units/µL, Qiagen, Hilden, Germany). To this master mixture 2,0 µL DNA-extract (tenfold diluted with Tris-EDTA buffer 1:10) is added to a total volume of 25,0 µL.
Initial denaturation and the denaturation step was carried out at 95 °C for 15 min and 30 s, respectively, elongation temperature was chosen in accordance with the primers melting temperatures, elongation took place at 72 °C for 30 s. The complete PCR-program was carried out for 27 to 30 cycles. Detection of PCR-products was accomplished using gel electrophoretic separation on polyacrylamide gels with subsequent visualisation with ethidium bromide.

2) Ruminants

DNA was isolated from 50 mg meat in each case applying the method according to (Binke et al., 2003). PCR was performed as described for poultry. PAGE was performed as described by Altmann et al. (2004) using the following molecular weight standards: λ-DNA/Eco RI + Hind III and pBR322/HaeIII. DNA amplification was carried out as described above. Primer design focusing on mitochondrial DNA was implemented by means of *HUSAR Data Base* (German Cancer Research Center, Heidelberg). The following primer pair was used for the amplification of a bison (bison bison) specific DNA fragment of a length of 138 bp: CY-Bis/forward: 5´ - AAATCCACTCAATACACCTCCC - 3´ (22 bp); CY-Bis/reverse: 5´ - CTAATCCTTGCCCTCATTCC - 3` (20 bp). In addition a buffalo specific primer system was applied resulting in a buffalo (bubalus bubalis) specific DNA fragment consisting of 242 bp: CY-Buf/forward: 5´ - TAGGCATCTGCCTAATTCTG - 3´ (20 bp); CY- Buf/reverse: 5´ - ACTCCGATGTTTCATGTTTCT - 3´ (21 bp).

III) Semi quantitative determination of goat tissue in meat products

After alignment of various sequences the 5´-flanking region of *beta-casein* (Roberts et al., 1992) appeared to be suitable for goat species identification, because of a 37 bp deletion in goat-DNA, compared to sheep-DNA. The specific primers BC290501 F (5´TCTGGTCCAATTGGTGAGAG 3´) and BC290501 R (5´AGGCCACAGGTGAAAAAGTC) were comercially synthesized by Qiagen (Hilden, Germany) and the amplified products were detected by means of a dual labeled probe BC290501 P (FAM-5´AGGGAAATGTTGAATGGGAAGGATATGC 3´-Tamra) as well as the SYBRE-Green 1 dye. For relative quantification two animal specific DNA fragments (97 bp and 154 bp) based on the myostatin gene can be used together with the above described goat specific primer/probe system (BC2900501). DNA was amplified in a 20 µl mixture containing 1x reaction buffer (Qiagen), 4.5 mM $MgCl_2$, 0.05 mM of each dNTP, 0.8 µM of each primer, 0.04 µM dual labelled probe (Qiagen) or 1 µl SYBR-Green 1 diluted 3000 fold, 1.25 units of HotStarTaq^{TM} DNA-polymerase (Qiagen) and 5 µl DNA solution diluted 40 fold. PCR program was as follows: 95 °C for 15 min; 95 °C for 30 s; 58 °C for 30 s; 72 °C for 30 s (SYBR-Green) and 65 °C for the pobe system. The determination of relative amounts of goat meat per total meat in meat products was calculated according to the modified delta-delta C_T-method.

Results and discussion

I) DNA isolation

The recoveries of extracted nucleic acid in self made reference meat products were determinated in dependence upon applied heating intensity. Up to a F_C value of 3.4 recoveries in a calculated range between 70 and 120 % were determinated. At higher heating intensities (F_C-values > 3.4) the nucleic acid yield is obviously decreasing. Nevertheless it can be said that by means of the optimized extraction system the main amount of nucleic acid can be isolated from processed meat products at high purity (A260 / A280).

II) Differentiation of animal species

1) Poultry

For all of the above mentioned domestic poultry species species specific primer systems based on the mitochondrial *cytochrome b* gene were adapted and developed, respectively. To verify the specificity the primer systems were tested against the examined domestic poultry species as well as against beef, bison,

sheep, goat, horse, kangaroo and ostrich. To prevent false positive results no-template-controls (NTC) were determined within each experiment. As an example in figure 1 the polyacrylamide gels visualised with ethidium bromide following PCR with the species specific primer system for pheasant-tissue are shown.

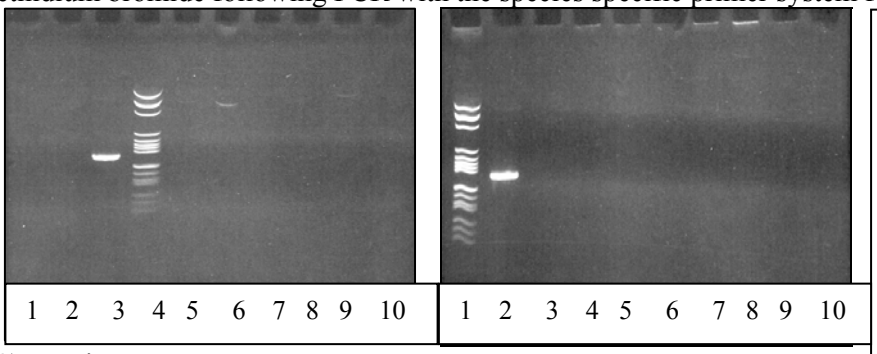

Figure 1: Specific detection of pheasant using the *cytochrome b* gene primer system for pheasant-tissue
Right: 1: marker pBR322, 2: pheasant, 3: guinea fowl, 4: quail, 5: chicken, 6: turkey, 7: goose, 8: duck, 9: pig, 10: NTC; Left: 1: cattle, 2: bison, 3: pheasant, 4: marker pBR322, 5: sheep, 6: goat, 7: horse, 8: ostrich, 9: kangaroo, 10: NTC

2) Ruminants

In order to identify the animal species and to investigate the specificity of the PCR system based on the *cytochrome b* gene for bison various animal species were tested by PCR for cross similarity (figure 2).

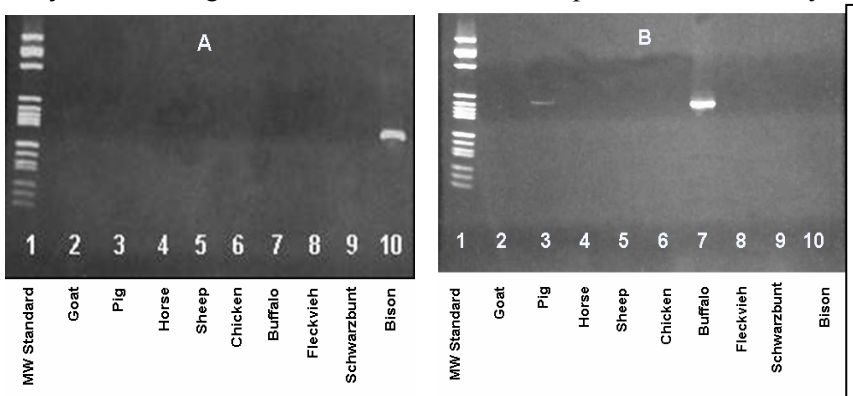

Figure 2: PAGE of various amplification products visualized by Ethidiumbromide intercalation: Gel A on the left shows only one distinct DNA band (lane 10) with 138 bp reflecting the specificity of the CY-Bis primer system for bison (bison bison) solely. There are no amplicons for tissue in the case of the other animals. On Gel B depicted on the right there is a clear DNA band (lane 7) with 242 bp for buffalo. This demonstrates the specificity of the CY-Buf primer system for buffalo (bubalus bubalis).

III) Semi quantitative determination of goat tissue in meat products

The resulting data show, that the probe assay represents a better comparability of the real and calculated content of goat meat in the products than the SYBR-Green I assay with a mean coefficient of varation (CV) of 20 %. The presented assays are suitable for a semiquantitative determination of goat meat in meat products. A quantitative determination with a small error of determination is only possible, if there is sufficient knowledge about the quality and purity of the DNA extracted from processed products. Nucleic acid can also be isolated from processed tissue at a high yield, but the quality of DNA, which is important for the efficiency of DNA amplification, is very much influenced by the applied heat treatment. The applied temperature and the time of heat treatment are decisive for the resulting DNA quality. The longer heat treatment and the higher the applied temperature during product processing are the more DNA degradation occurs. Therefore quantification procedures demand suitable reference standards with comparable composition and processing procedure.

Furthermore the varying DNA content in different animal tissues has to be taken into consideration. Not heated minced meat shows a DNA content of 0.3 – 0.5 g/kg, whereas liver has a tenfold higher content of DNA. However, the DNA content of fatty tissue is only around 0.1 g/kg.

The use of system BC290501 in proper combination with the two possible myostatin gene fragments is of importance with respect to accuracy and reproducibility for quantification. The presence of poultry and kangaroo in the products has to be taken into consideration (Binke et al., 2005).

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