PCR analysis for meat products authenticity – detection of horse meat

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Abstract. Food adulteration regarding species origin of meats is a common problem in the meat products sector. With regard to horse meat, its undeclared use in food products is not only a fraud, but could present a health risk since is often associated with the presence of the veterinary drug phenylbutazone in meat products. Therefore, it is important to use reliable methods for authentication of meat products regarding their species composition, which are applicable to complex food matrices. Polymerase chain reaction (PCR) with species-specific primers remains the most widely used analytical approach to detect species-related food adulteration due to its high sensitivity and specificity. The aim of the present study was to establish the authenticity of 20 different meat products on the Bulgarian market without declared horse meat content by using a species-specific PCR method. The specificity test of the PCR method used showed no amplification of DNA from beef and pork. A detection limit of 0.01% horse DNA in three-component meat mixtures was established for the PCR method. The PCR method enabled detection of undeclared presence of horse meat in 25% of the analyzed meat products, which demonstrates the need for strict control regarding authenticity in the meat food chain.

1 Introduction

Adulteration of meat products is a global issue with a constantly rising number of detected incompliances in various aspects, including species identity [1, 2]. The latest report of the EU platform Administrative Assistance and Cooperation System – Food Fraud, which was launched in 2015, shows a constantly rising number of requests for food fraud investigation [3]. The scale of illegal use of horse meat in meat products has triggered a special focus of the targeted Europol action OPSON IX in 2020, aiming to support the national authorities within the EU to combat fraud with horse meat. Horse meat can easily be mixed with pork, bovine and sheep meats in processed meat products because of their similar pigmentation and this fraud cannot be detected organoleptically by the consumer [2]. Food labelling regulations require the identity of meat in meat products to be accurately labelled in order to facilitate the informed consumer's choice [4]. Therefore, the undeclared use of horse meat is a quality issue (incompliance) and a legal fraud. On the other hand, undeclared horse meat could also present a health risk since is often associated with the presence of the veterinary drug phenylbutazone in meat products [5]. Therefore, there is a growing need for strict control regarding the movement of meat species in the food chain and the composition of meat products, which can only be ensured by the use of reliable methods for authentication which are applicable to complex food matrices.

A range of analytical approaches for identification of meat species in raw meat includes sensory analysis, anatomical differences, histological differentiation, properties of tissue fat and level of glycogen in muscle tissue, as well as more contemporary protein based and DNA based techniques [6 - 8]. Protein-based methods have high sensitivity for unprocessed meat products but they are not very suitable when applied to processed meats, because of protein denaturation [2]. After the 2013 horse meat scandal in the EU, authorities and researchers focused their efforts on the development of reliable authentication methods and many techniques based on DNA analysis were adopted, with special attention on the polymerase chain reaction (PCR) method due to its high sensitivity and specificity, which allow the detection of very small amounts of DNA in raw materials and processed foods [9, 10]. One of the most commonly used PCR approach in food authentication is PCR with species-specific primers, followed by signal detection with gel electrophoresis [8]. The number of published studies on food authentication regarding the presence of horse meat using modern techniques is relatively limited. Therefore, the aim of the present study was to apply a species-specific PCR method for screening of different types of processed meat products in order to establish their authenticity in terms of undeclared presence of horse meat.

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2 Materials and methods

2.1 Analysed samples

The analysed samples were purchased from local supermarkets and included 20 meat products with no declared horse meat content. They were divided into four groups: 9 fresh processed meat products, 3 cured meat products, 6 raw-cooked meat products and 2 other meat products. Horse (*Equus caballus*), bovine (*Bos tauros*) and porcine (*Sus scrofa*) muscle tissues were used as positive and negative control samples. All meat products were stored at -20℃ until analyzed.

2.2 Preparation of meat mixture

Three-component meat mixtures (horse, bovine and porcine) were prepared using raw meats from the target species. Seven standards of meat mixtures were prepared to a final weight of 100 g, containing 10, 5, 1, 0.5, 0.1, 0.05 and 0.01 % (w/w) of horse meat in bovine + pork meat (1:1).

2.3 DNA extraction

All samples were homogenized with a laboratory homogenizer VWR 431 (VWR, USA). DNA from the samples, controls and meat mixtures was extracted according to the modified CTAB method [10]. The DNA concentration was determined by measuring the absorbance at 260 nm. The purity of DNA extracts was calculated by the ratio of the absorbance at 260 nm and 280 nm (Shimadzu UV-VIS, Shimadzu Corporation, Japan). The final DNA concentration was adjusted to 50 ng/µl. Each sample, control and meat mixture were extracted in three replicates.

2.4 PCR amplification of horse DNA

Species-specific PCR amplification of horse DNA was conducted with primers Horse_F (5'-CTA TCC GAC ACA CCC AGA AGT AAA G-3') and Horse_R (5'-GAT GCT GGG AAA TAT GAT GAT CAG A-3') [11], obtained from Metabion (Martinsried, Germany). The primer pair gives the expected fragment size of 153 bp.

The PCR reaction was performed in a final volume of 20 µl, containing 1 µl of DNA (50 ng), 0.5 µM of each primer and 8 µl of Red-Taq DNA Polymerase Master Mix (Canvax Biotech, S.L., Spain). The amplification was carried out in a PCR 2720 Thermal Cycler (Applied Biosystems, USA) using the following program: initial denaturation at 95℃ for 5 min, followed by 35 cycles of denaturing at 95℃ for 1 min, annealing at 61℃ for 1 min, extension at 72℃ for 1 min, and final extension at 72℃ for 7 min. PCR products were visualized in 1% agarose gel stained with SafeView (NBS Biologicals, Huntingdon, England) at 100 V for 50 min using VWR Mini Electrophoresis System (VWR, Germany) and MiniBis Pro (DNR Bio-Imaging Systems, Israel) for gel visualization.

2.5 Specificity and sensitivity of the species-specific primers

The specificity of the selected primers was confirmed by amplification of 50 ng/µl DNA extracts from horse muscle tissue, bovine muscle tissue and porcine muscle tissue. The sensitivity of the primers was determined by amplification of DNA extracts from the seven standard meat mixtures, containing different amounts of horse meat (10, 5, 1, 0.5, 0.1, 0.05 and 0.01 % (w/w), respectively).

3 Results

3.1 DNA extraction, concentration and purity

The results from the spectrophotometric measurement of DNA concentration and purity are summarized in Table 1.

Table 1. Concentration and purity of DNA extracts from raw and processed meat products

No	Sample	C_{DNA}, ng/µl ± SD	A_{260}/A_{280} ± SD
1	Fresh processed meat products (9)	207.42 – 748.38	1.68 – 1.83
2	Cured meat products (3)	380.87 – 597.52	1.74 – 1.81
3	Raw-cooked meat products (6)	322.05 – 516.19	1.67 – 1.79
4	Other meat products (2)	609.69 – 677.71	1.72 – 1.77

DNA concentration differed according to the type of meat products. Highest values were obtained for the group of fresh processed meat products – between 207.42 ng/µl and 748.38 ng/µl. Similar results were received for the other three groups of meat products (Table 1). The purity of the extracts obtained from all groups of analyzed samples was high (A_{260}/A_{280} of 1.67 to 1.83 for all extracts). This shows that CTAB extraction method provided adequate purification from different kinds of contaminants such as polysaccharides, polyphenols and proteins.

3.2 Specificity and sensitivity testing of species-specific primers

Specificity testing of the selected primers is of key importance in order to ensure the detection of horse DNA in different meat products (Fig. 1). Experimental data demonstrated the presence of a specific fragment with size of 153 bp only in the DNA extract from horse muscle tissue, which showed the absence of nonspecific amplification and confirmed the specificity of the PCR method.

The sensitivity of the PCR analysis for horse DNA detection was evaluated by amplification of seven standard meat mixtures, containing different amounts of horse DNA (Fig. 2).

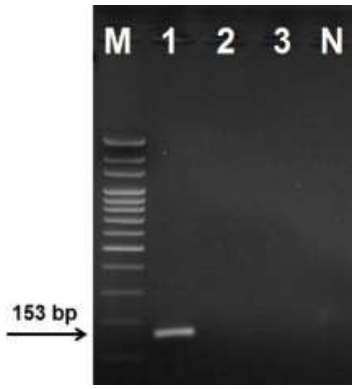

Fig. 1. Agarose gel electrophoresis of PCR products of DNA extracts from muscle tissue samples. P: positive control; 1: DNA from horse muscle tissue; 2; DNA from bovine muscle tissue; 3: DNA from porcine muscle tissue; N: negative PCR control

Data presented in Fig. 2 demonstrated successful amplification of a specific fragment of 153 bp in DNA extracts from the positive control as well as in all seven standard meat mixtures. Based on the obtained results, the concentration of 0.01 % horse DNA was determined as the limit of detection (LOD) of the species-specific PCR method. A PCR fragment of 153 bp was not detected in the negative PCR control.

3.4 Application of PCR assay for screening of meat products with no declared horse meat content

DNA extracts from all analyzed samples were subjected to PCR analysis of horse DNA for food authenticity screening. The chosen species-specific primers successfully amplified the 153 bp fragment of the mitochondrial genome. The obtained data is presented in Figure 3. Results demonstrated that horse DNA was found in three samples from the group of fresh processed meat products (Fig. 3A), one of the raw-cooked meat products (Fig. 3B) and one sample from the other meat products (Fig. 3D).

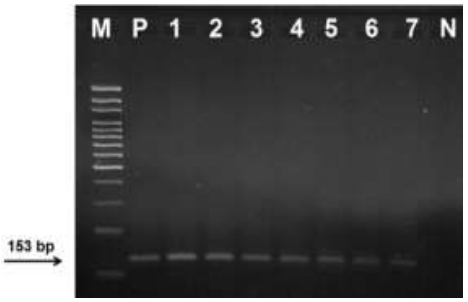

Fig. 2. Agarose gel electrophoresis of PCR products of DNA extracts from seven standards, containing different amounts of horse DNA. M: 100 bp ladder; P: positive control; 1: 10% of horse DNA; 2: 5% of horse DNA; 3: 1% of horse DNA; 4: 0.5% of horse DNA; 5: 0.1% of horse DNA; 6: 0.05% of horse DNA; 7: 0.01% of horse DNA; N: negative PCR control

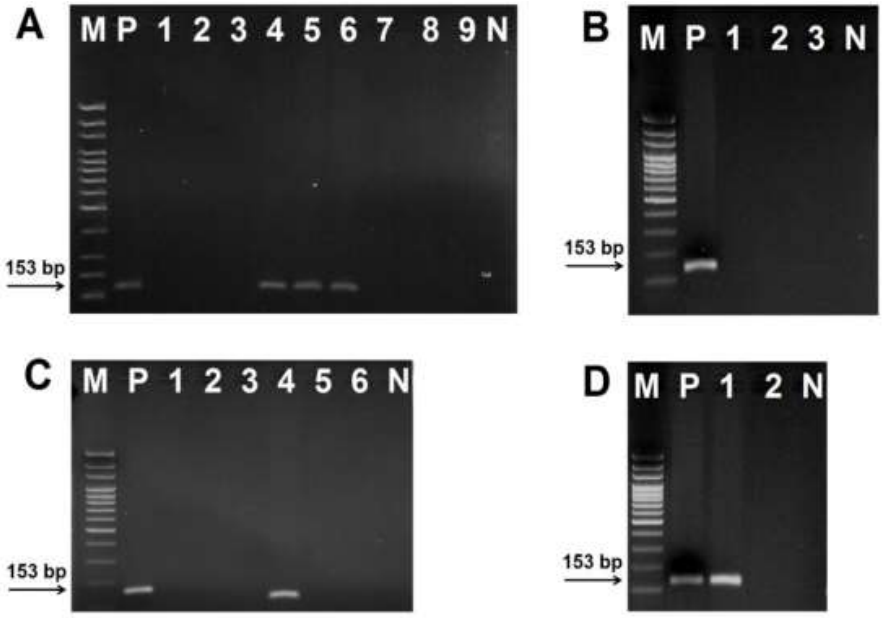

Fig. 3. Agarose gel electrophoresis of PCR products of DNA extracts from fresh processed meat products (A), raw-cooked meat products (B), cured meat products (C) and other meat products (D) for detection of horse DNA. M: 100 bp ladder; P: positive control; 1 – 9 (A), 1 – 6 (B), 1 – 3 (C), 1 – 2 (D): meat products; N: negative PCR control

A specific fragment with size of 153 bp was not detected in any of the cured meat products (Fig. 3C). No amplification was observed in negative PCR control, which demonstrated the absence of non-specific amplification as well as the purity of the components in the PCR analysis. The results from the study show that horse DNA was detected in five of the samples, which represented 25 % of the analyzed meat products (Fig. 4).

4 Discussion

4.1 DNA extraction, concentration and purity

Extraction of high quantity and intact DNA is of key importance for food authenticity assays. Although DNA concentrations differed according to the type of meat

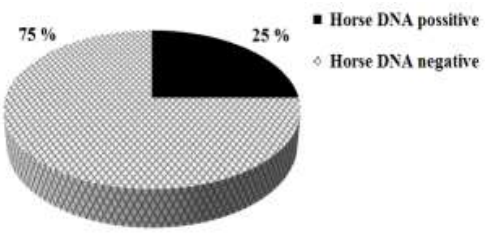

Fig. 4. Food authenticity screening for horse DNA in meat products without horse meat listed on the product labels

products, the obtained DNA extracts from all groups of meat products were with high concentration values (Table 1). The obtained data for purity of the extracts showed that CTAB extraction method provided adequate purification from different kinds of contaminants such as polysaccharides, polyphenols and proteins. These results were in accordance with other authors who verified the good performance of the CTAB method for complex food matrices and highly processed food products [12, 13].

4.2 Specificity and sensitivity of the species-specific primers

In the present study, a species-specific PCR method was applied for screening of different types of meat products in order to establish their authenticity regarding the presence of horse meat. The specificity test is essential for the clear differentiation of horse from other meats in raw and processed meat products. Our data (Fig. 1) showed no cross-reactivity with DNA from other species as bovine and porcine. These results confirmed previous studies which were conducted with the same species-specific primers [11, 14].

A sensitive PCR assay can be established when the target sequence is a multicopy gene, such as a mitochondrial gene [15]. Based on the results obtained from the sensitivity test of the species-specific PCR assay (Fig. 2), the LOD of the method was established at 0.01 % of horse DNA in triple meat mixtures. This result is higher than the values reported by Kesmet et al. [11], who achieved a LOD of 0.1 % of foreign DNA. Abd El-Razik et al. [16] also achieved an LOD of 0.01 % horse meat in beef products. This low value corresponds to LOD obtained by other authors using real-time PCR analysis [17], which confirms the excellent sensitivity of the currently used method.

4.3 Application of PCR assay for screening of meat products with no declared horse meat content

Food authenticity and traceability are significant problems in the meat chain due to common fraudulent practices. After the 2013 horse meat scandal in the EU, the undeclared horse meat use in meat products became a specially targeted issue by the regulators, control authorities, the food industry, the market players and the consumers. This triggered an urgent need of highly sensitive, robust and reliable analytical methods for horse meat detection. Our literature survey found only several studies on this issue. Therefore, the present study aimed to evaluate the efficiency of a designed species-specific PCR method for the detection of horse meat in variously processed meat products. The selected samples included 20 meat products with no declared horse content and were divided into four groups – fresh processed meat products, cured meat products, raw-cooked meat products and other meat products. Results showed the presence of horse DNA in 25% of the analysed samples (Fig. 3) which demonstrates the necessity of strict authenticity control in the meat food chain. Abd El-Razik et al. [16] tested 96 meat samples and reported lower percentage of mislabelling (only two sausage samples were contaminated with horse meat). On the other hand, Mousa et al. [18] revealed that the incidence of adulteration in 100 analysed meat products with horse meat was 38 % in Alexandria city of Egypt. Chisholm et al. [8] performed horse presence assays on commercial samples including cured meats: salami and ham, but the study was carried out only with samples with declared horse content. Results from the present food authenticity study show the same level of fraudulent samples as a survey of the Food Safety Authority of Ireland (FSAI) carried out in 2013 [19]. FSAI analysed 22 burger samples with no declared equine content on the label and established that 27.3 % of the samples gave positive result for the presence of equine meat. All reported studies, including the present work, demonstrate the significant scale of fraudulent practices with undeclared horse meat use, which indicates that strict analytical control must be applied throughout the food chain in order to ensure food authenticity and safety.

5 Conclusions

The present work considered the application of a species-specific PCR method for the detection of horse meat in raw and processed meat products at the Bulgarian market. The applied PCR assay is characterized by high sensitivity and specificity. Results from the screening demonstrated the significant levels of undeclared horse meat use and the need for more stringent analytical control in order to prevent food adulteration and ensure food quality and safety. The applied species-specific PCR assay proved to

be a comparatively inexpensive and rapid method for horse meat detection and could be successfully applied for routine analyses of raw and processed meat products.

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