

A duplex PCR method for authentication of beef and pork content in raw and processed meat products

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Abstract. The regulatory framework regarding food labelling aims to ensure food safety and prevent consumer misleading and food fraud. However, legal compliance cannot be controlled without the use of reliable analytical methods. Polymerase chain reaction (PCR) with species-specific primers is considered the most reliable analytical tool for species identification in various raw and processed foods. Duplex PCR methods allow the simultaneous detection of two species within a product, which may considerably reduce analytical time, effort and price. Therefore, the objective of our study was to develop a duplex PCR method for detecting beef and pork in raw and processed meat products. Our research resulted in elaborating a highly specific method, with LOD for each sequence of 0.01 % target DNA in two-component meat mixtures. The application of the duplex PCR for authentication of raw and processed meat products on Bulgarian market showed that 45% of the analysed meat products had labelling incompliances regarding the content of beef and pork. These results demonstrate the need for more stringent and authenticity control in the meat processing sector and at the market by using rapid and reliable analytical methods in order to reduce the current levels of food fraud and protect consumer's rights.

1 Introduction

Food fraud has been an issue for food production and trade since early ages, and despite efforts to prevent it at a global scale, it continues to cause losses for the global food industry estimated to approximately US \$40 billion annually [1]. It is also the major reason for loss of consumer confidence in foods at the market and for economic disadvantages to various stakeholders in the food supply chain. The global issues with fraudulent practices in the past 15 years (e.g. replacement of horse meat in beef products in 2013) resulted in a world-wide consensus for an urgent need to develop methods and approaches to tackle food fraud and ensure food authenticity.

Meat products are a major food group associated with numerous food fraud issues, therefore wide research efforts are focused on the development of rapid and reliable authentication methods. Detection of adulteration of meat products is necessary for legal, economic, religious and health reasons [2]. Recently, there have been increasing concerns about the adulteration of meat products with those of other species, such as pork and beef [3].

Food authenticity assays of species identification include chromatographic, immunological, electrophoretic and genetic methods [2, 4]. Some of these methods are applicable only for raw materials, while others can be used for species identification in high processed food products

as well [5-7]. In comparison with protein, DNA is more stable and resistant to such factors as high temperature, pressure, and chemical compounds [8-9]. The polymerase chain reaction (PCR) method is one of the most common methods in authentication analyses due to its high sensitivity, specificity and relatively short period of time necessary for assays [6, 9]. Multiplex PCR methods based on simultaneous amplification of multiple sequences save considerable time and effort by decreasing the number of reactions required to assess the possible presence of different species in meat products [10]. The objective of the present study was to develop a duplex PCR method for identification of beef and pork species which may be applied for the authentication of raw and processed meat products.

2 Materials and methods

2.1 Analysed samples

The samples used in this study included 20 meat products, which were divided into two groups – raw meat products (9 samples) and high processed meat products (11 samples). Bovine (*Bos taurus*), porcine (*Sus scrofa*) and chicken (*Gallus gallus*) muscle tissues were used as positive and negative controls. All meat products and controls were obtained from Bulgarian supermarkets and were stored at -20°C .

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2.2 Preparation of meat mixture

Two independent two-component meat mixtures (bovine in porcine and porcine in bovine) were prepared using raw muscle tissues from the target species. For each meat mixture, seven standards 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 beef meat in porcine and the same percentage (w/w) of pork meat in bovine, respectively. In addition, three-component meat mixtures were used as standards for evaluation of LOD of the duplex PCR method. Each standard was prepared to a final weight of 100 g, containing equal amounts of beef and pork meat in chicken meat in different percentages (10, 5, 1, 0.5, 0.1, 0.05 and 0.01 % (w/w)).

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 [11]. 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/ μ l. Each sample, control and meat mixture was extracted in three replicates.

2.4 Selection of species-specific primers

Species-specific primers for amplification of beef and pork DNA were designed according to Köppel et al. [12] and Lahiff et al. [13] and obtained from Metabion (Martinsried, Germany). The primer pairs amplified PCR fragments of 96 bp and 212 bp for beef and pork DNA, respectively.

2.5 Duplex PCR conditions

Series of experiments were conducted in order to determine the optimal conditions for duplex PCR. Additionally, the temperature and duration of the particular stages of the PCR reaction were adapted. Duplex PCR was carried out with a PCR 2720 Thermal Cycler (Applied Biosystems, USA). The PCR reaction was performed in a final volume of 20 μ l, containing 1 μ l of DNA (50 ng), 0.4 μ M of each primer and 8 μ l of Red-Taq DNA Polymerase Master Mix (Canvax Biotech, S.L., Spain).

Parameters of amplification were as follow: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturing at 95°C for 1 min, annealing at 61°C for 45 s, extension at 72°C for 1 min, and final extension at 72°C for 7 min. Each extract was amplified in duplicate assays. Further the amplified fragments were analysed by electrophoresis in a 2% agarose gel carried out in 0.5xTBE buffer for 60 min at 100 V, stained with Safe View Nucleic Acid Stain (NBS Biologicals, England). The agarose gel

was visualized under UV light using MiniBIS Pro transilluminator and gel documentation system (DNR Bio-Imaging Systems, Israel).

2.6. Specificity and sensitivity of simplex and duplex PCR assays

The specificity of simplex and duplex PCR assays was confirmed by amplification of 50 ng/ μ l DNA extracts from bovine and porcine muscle tissues. The sensitivity of PCR methods was determined by amplification of DNA extracts from the two- and three-component meat mixtures, described in section "Preparation of meat mixtures".

3 Results and discussion

3.1 DNA extraction, concentration and purity

Quality and yield of the isolated DNA are critical factors in authentication of raw and processed meat products. The results from the spectrophotometric measurement of DNA concentrations shown that concentrations varied between 207.42 – 748.38 ng DNA/ μ l DNA extract for raw meat products. Although DNA concentration differed according to the type of meat products, similar results were obtained for the group of high processed meat products (322.05 – 677.71 ng DNA/ μ l DNA extract) (data not shown).

DNA purity of the extracts from all analyzed samples was high – $A_{260}/A_{280} = 1.68 - 1.83$ for raw meats and $A_{260}/A_{280} = 1.67 - 1.81$ for high processed meat products (data not shown). The obtained data demonstrated that the CTAB extraction method produced high quality DNA extracts. The high performance of the CTAB method for complex food matrices and highly processed food products was in agreement with other researchers [14-16].

3.2 Specificity of simplex and duplex PCR assays

The specificity of simplex PCR methods was assessed by amplification of 50 ng/ μ l DNA extracts from bovine and porcine muscle tissues (Figure 1). Results presented in Figure 1A demonstrated the presence of a specific 96 bp PCR fragment only in the DNA extract from bovine muscle tissue. The absence of a specific band in DNA extracts from porcine muscle tissue and the negative PCR control confirmed the specificity of the method. Similarly, the presence of a specific fragment with the size of 212 bp only in the DNA extract from pork meat when using porcine-specific primers, showed the good performance of the method and the lack of cross reactivity with the DNA from bovine muscle tissue (Figure 1B). This experimental data was in agreement with Köppel et al. and Lahiff et al. who reported the high specificity of the primers used [12-13].

The results from the specificity test of the developed duplex PCR method are presented in Figure 2.

Amplification was observed only with the target species present. No cross reactivity was detected when other species were present and in the negative PCR control.

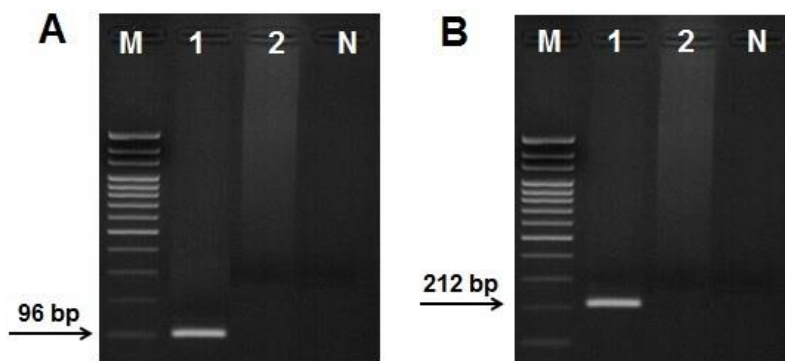


Fig. 1. Agarose gel electrophoresis of PCR products of DNA extracts from bovine and porcine muscle tissues with bovine-specific primers (A) and porcine-specific primers (B). (A) – M: 100 bp ladder; 1: DNA extract from bovine muscle tissue; 2: DNA extract from porcine muscle tissue; N: negative PCR control. (B) – M: 100 bp ladder; 1: DNA extract from porcine muscle tissue; 2: DNA extract from bovine muscle tissue; N: negative PCR control.

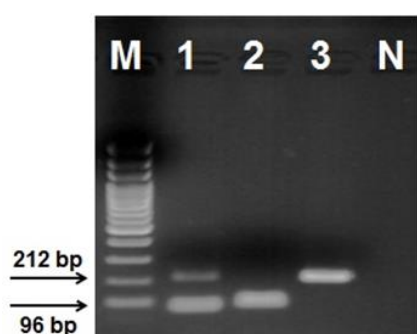


Fig. 2. Agarose gel electrophoresis of PCR products of DNA extracts from muscle tissue samples. M: 100 bp ladder; 1:DNA extract from meat mixture (beef:pork = 1:1); 2: DNA extract from bovine muscle tissue; 3: DNA extract from porcine muscle tissue; N: negative PCR control.

3.3 Sensitivity of simplex and duplex PCR assays

The sensitivity of the simplex PCR methods for detection of beef and pork DNA was evaluated by amplification of seven standards, containing different amounts of target DNA (10, 5, 1, 0.5, 0.1, 0.05 and 0.01 %, respectively). The experimental data is presented in Figure 3, demonstrating the successful amplification of a specific 96 bp PCR product in the DNA extracts from the positive control and all seven standards (Figure 3A). The same result was observed with the sensitivity test of PCR for pork DNA detection. The data in Figure 3B shows the presence of amplified fragments with size of 212 bp in positive control and all standards. Based on the obtained results, the limit of detection (LOD) of the simplex species-specific PCR methods was 0.01 % of beef and pork DNA (Figure 3A and 3B, respectively).

PCR fragments with the corresponding size were not detected in any of the negative PCR controls. The obtained results are in agreement with Köppel et al. who reported similar LOD with real-time PCR analysis [12]. On the other hand, the obtained LOD was higher than the reported values by Kesmet et al. and Laube et al., who achieved a LOD of 0.1 % of foreign DNA [2, 17].

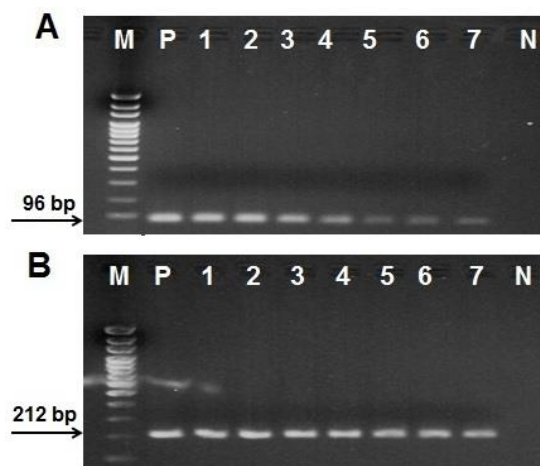


Fig. 3. Agarose gel electrophoresis of PCR products of DNA extracts from seven standards, containing different amounts of beef DNA (A) and pork DNA (B). (A) – M: 100 bp ladder; P: positive control; 1: 10% of beef DNA; 2: 5% of beef DNA; 3: 1% of beef DNA; 4: 0.5% of beef DNA; 5: 0.1% of beef DNA; 6: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

Further, a sensitivity test of the developed duplex PCR method was conducted (Figure 4). The results presented in Figure 4 demonstrate the successful amplification of both DNA target sequences in DNA extracts from the positive control and all standards. Based on the obtained results, the concentration of 0.01 % target DNA was determined as LOD of the duplex PCR method. It is important to note that the sensitivity of the duplex PCR method was the same as the sensitivity of the simplex methods for detection of beef and pork DNA. The results presented in Figure 4 demonstrate the successful amplification of both DNA target sequences in DNA extracts from the positive control and all standards.

Identical results were obtained from other authors when evaluating the sensitivity of multiplex PCR methods for detection of species-specific DNA [18-19]. On the other hand, the duplex PCR method developed in the

present study was characterized with higher sensitivity compared to Ilhak et al., who estimated a detection limit of 0.5 % foreign DNA [20].

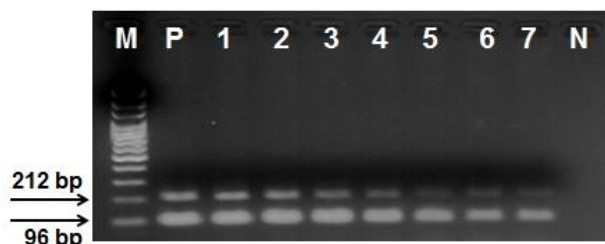


Fig. 4. Agarose gel electrophoresis of PCR products of DNA extracts from seven standards, containing equal amounts of beef and pork meat in chicken meat. M: 100 bp ladder; P: positive control; 1: 10% of each DNA; 2: 5% of each DNA; 3: 1% of each DNA; 4: 0.5% of each DNA; 5: 0.1% of each DNA; 6: 0.05%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

3.4 Application of the duplex PCR method for authentication of raw and processed meat products

Nowadays meat products are globally distributed due to advances of the cold chain and the technological advances resulting in improved shelf life. The correct labelling of meat product is very important for ensuring consumers' trust and confidence. In order to assess the applicability of the developed duplex PCR method for food authenticity

screening in the meat product chain, it was used to analyse 20 raw and processed meat products with regards to beef and pork content. The results from this screening study are presented in Figure 5. The duplex PCR assay showed the presence of beef and pork DNA in all analysed raw meat products (Figure 5A). PCR fragments with size of 96 bp were detected in nine of the processed meat products, while fragments, corresponding to beef DNA were found in seven of the samples (Figure 5B). These results demonstrate the good performance of the duplex PCR method when applied to raw (Figure 5A) and processed meat products (Figure 5B). Fragments of 96 bp and 212 bp, corresponding to the target DNA sequences were not observed in the 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 showing mislabelling of the analysed meat products are presented in Table 1. As illustrated, five of the raw meat products (samples 1 to 9) contained undeclared pork ingredients. Similarly, undeclared beef meat was found in one sample (sample 6). The percentage of mislabelling was lower for the group of processed meat products, where unlisted pork meat was found only in one sample (sample 12). The same situation was observed for unlabelled beef content (sample 14). It was interesting to note that pork meat ingredients were listed on the label of sample 20, however the duplex PCR assay did not show presence of pork. In summary, nine of the meat products, which represent 45 % of the analyzed samples, had a labelling incompliance.

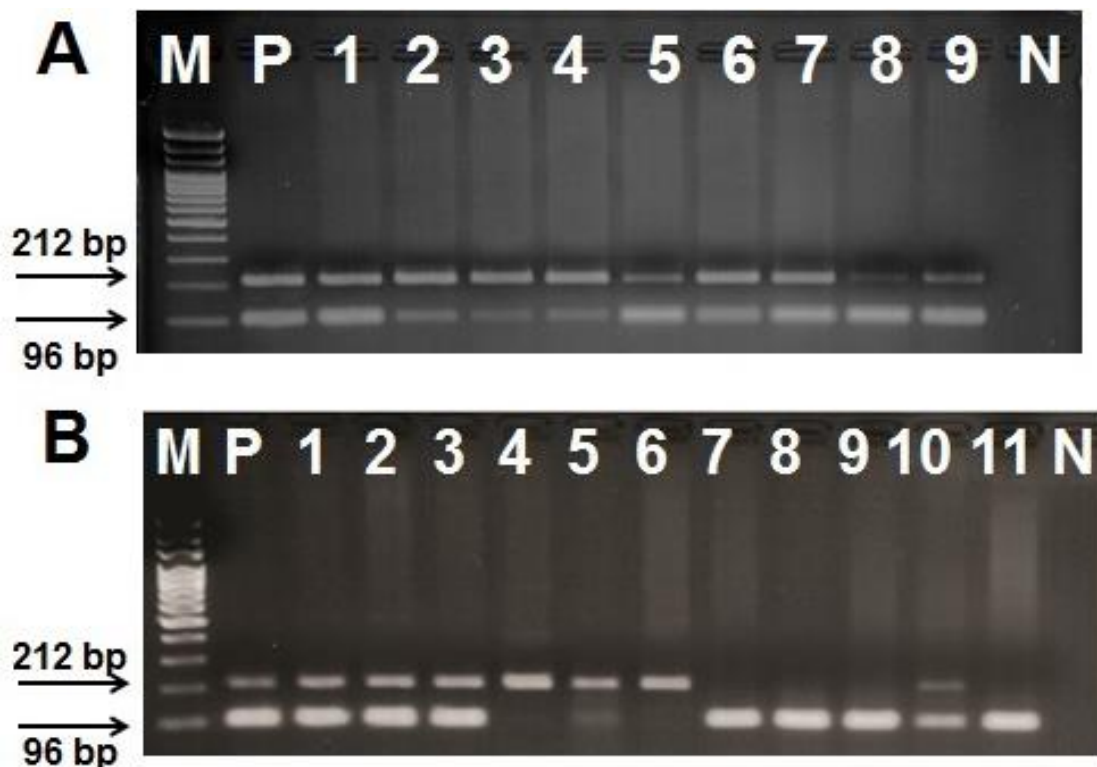


Fig. 5. Agarose gel electrophoresis of PCR products of DNA extracts from raw meat products (A) and processed meat products (B). (A) – M: 100 bp ladder; P: positive control; 1 - 9: raw meat products; N: negative PCR control. (B) – M: 100 bp ladder; P: positive control; 1 - 11: processed meat products; N: negative PCR control.

Table 1. Application of the developed duplex PCR method for authenticity screening of raw and processed meat products with regards to beef and pork content

Sample №	Beef		Pork	
	Label	PCR	Label	PCR
1	+	+	+	+
2	+	+	+	+
3	+	+	-	+
4	+	+	-	+
5	+	+	-	+
6	-	+	+	+
7	+	+	-	+
8	+	+	-	+
9	+	+	+	+
10	+	+	+	+
11	+	+	+	+
12	+	+	-	+
13	-	-	+	+
14	-	+	+	+
15	-	-	+	+
16	+	+	-	-
17	+	+	-	-
18	+	+	-	-
19	+	+	+	+
20	+	+	+	-

Similar results were obtained by other authors. Cai et al. tested 55 different meat products and reported that 31% of the samples contained undeclared meat ingredients [18]. The presented data was in agreement with Di Pinto et al., who analyzed 72 meat products and revealed a high rate of mislabeling cases (57%) [21]. Significantly higher percentage of mislabelling was established by the Food Safety Authority of Ireland (FSAI). The Agency analysed 22 burger samples with no declared pork content on the labels and reported that 81.8% of the samples gave positive result for the presence of porcine DNA [22]. These results demonstrate that the incidence of meat fraud is still widely spread, which stresses the necessity of strict authenticity control in the meat food chain.

4 Conclusions

The present work was focused on the development of a duplex PCR method for identification of bovine and porcine DNA and on testing its performance in authentication of raw and processed meat products. The PCR assay is characterized by high sensitivity and specificity. The obtained results revealed that commercial meat products are frequently adulterated, which clearly demonstrated the necessity of strict food authenticity control. The developed duplex PCR assay is rapid and economically efficient and may be successfully applied for routine analyses aiming the authentication of raw and processed meat products regarding meat species.

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