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Efficiency of PCR-RFLP and Species-specific PCR for the Identification of Meat Origin in Dry Sausages

Ivona DJURKIN KUŠEC[1], Danijela SAMAC[1], Vladimir MARGETA[1], Žarko RADIŠIĆ[1], Dragutin VINCEK[2] and Goran KUŠEC[1]*

[1]*Faculty of Agriculture, J.J. Strossmayer University of Osijek, Osijek, Croatia;*
[2]*Department of Agriculture, Varaždin County, Varaždin, Croatia*
**Corresponding author: idurkin@pfos.hr*

Abstract

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The purpose of this investigation was the identification of chicken, beef and sheep meat in pork sausages using PCR-RFLP and PCR with species-specific primers. Six dry fermented pork sausages were produced by adding beef, sheep and chicken meat to each in the amount of 1 and 5%. DNA was extracted from five regions of each sausage and PCR-RFLP together with PCR using species-specific primers was performed. PCR-RFLP analysis was successful only for chicken meat, while species-specific PCR was effective for identification of chicken, beef and sheep meat in all ratios and from all regions of the sausages. The results of our study show that discovering adulteration using PCR-RFLP is suitable only for chicken meat in the investigated products, while for detection of beef and sheep meat use of species-specific oligonucleotides is more effective.

Keywords: DNA; traceability; meat species; dry fermented product

Humans have always consumed meat in various forms, either after thermal processing or prepared in a way that allows long-term storage, such as in the form of dry sausages. Over the years these products have become delicacies, and in many European countries they are protected by PDO (Protected Designation of Origin) or PGI (Protected Geographical Indication) trademarks. These products are sold for high prices on the market, and for this reason are an attractive target for meat adulteration. The methods for identification of animal origin are based on electrophoresis, isoelectric focusing, chromatography, DNA hybridisation, polymerase chain reaction (PCR), the enzyme-linked immunosorbent assay and SDS-PAGE (Kumar *et al.* 2013). Among them, PCR techniques are particularly attractive as they are quite simple, fast and cheap. Furthermore, DNA is relatively stable, meaning it can be isolated from fresh or frozen as well as processed, degraded and mixed products. DNA techniques include PCR with species-specific primers (Meyer *et al.* 1995; Matsunaga *et al.* 1999; Maede 2006; Doosti *et al.* 2014), restriction fragment length polymorphism (PCR-RFLP) (Haider *et al.* 2012), randomly amplified polymorphic DNA (RAPD) (Arslan *et al.* 2005), amplified fragment length polymorphism (AFLP) (Martinez & Yman 1999), terminal restriction fragment length polymorphism (Terminal-RFLP) (Wang *et al.* 2010) and quantitative PCR (qPCR) assays (You *et al.* 2014). All these techniques have their advantages but also limitations. This is especially true for meat mixtures, mainly in fermented products where DNA is highly

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prone to degradation due to changes in pH, temperature and other technological operations. After the horsemeat scandal in 2013, a control plan aimed at discovering the adulteration of meat containing beef has been implemented. Moreover, in 2015, the European Commission organised a control plan to assess the prevalence of white fish mislabelling in the EU, as fishery products were identified as high-risk goods for species substitution. Following on from the results of these two coordinated plans, more studies on meat adulteration, especially in products achieving high prices on the market, can be expected. According to EU Commission recommendation 2014/180/EU and the Commission Recommendation of 12.3.2015 on establishing a prevalence of fraudulent practices in the marketing of foods, all samples should be submitted to an initial screening test, while the choice of the test is left to the member state. In the case of raw meat, protein-based methods are a good choice as they can detect minute amounts of foreign meat added to the product (Asensio *et al* 2008; Di Giuseppe *et al.* 2015). However, proteins denature in processed products (subjected to cooking, smoking, salting etc.), which results in a failure to detect foreign meat in investigated products.

Therefore, the aim of this paper was to evaluate the efficiency of PCR-RFLP and species-specific PCR as potential screening test methods for identification of chicken, beef and sheep meat in heterogeneous meat mixtures with pork subjected to different processing operations, such as dry fermented sausages.

MATERIAL AND METHODS

The study was conducted on six traditional Croatian dry sausages. Normally, the sausage is produced from pork, salt, garlic, ground red paprika and pepper, filled into pig *caecum* and exposed to smoking, drying and ripening for at least three months. For the purpose of this investigation, the sausages were produced as specified, but in three sausages pig meat was substituted with 5% and in three sausages with 1% chicken, beef and sheep meat. It is known that during mixing the components become distributed through the sausage in an unpredictable manner, meaning that such small amounts of foreign meat could be distributed for example only in the middle or at one end of the sausage. For that reason, each sausage was cut at five sites and samples for DNA analysis (2 g each) were taken from each of the regions. Samples were homogenised using a mortar and pestle and DNA was extracted using the DNeasy Mericon Food Kit (Qiagen, Germany), as it was shown to be most suitable for the analysis of these kinds of samples (Djurkin Kušec *et al.* 2015). Concentration and purity of the DNA were determined using the Nanophotometer® UV/VIS spectrophotometer (IMPLEN GmbH, Germany). For the PCR-RFLP analysis, a set of universal primers were used (CYTB1 5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3'; CYTB2 5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3') for the amplification of the CYTB gene region (Meyer *et al.* 1995). PCR was performed using SapphireAmp® Fast PCR Master Mix (Takara Bio, Inc., Japan) in a 25 µl reaction volume containing 12.5 µl master mix, 9.5 µl water, 5 pmol of each primer and 180 ng of DNA. The PCR was performed on a thermocycler (Eppendorf Mastercycler Gradient; Eppendorf AG, Germany) under the following conditions: 40 cycles of denaturation at 98°C (10 s), annealing at 55°C (30 s), and extension at 72°C (1 min). Obtained PCR products were electrophoresed on 1.5% 0.5 × TBE agarose gels stained with GelRed™ Dropper (Olerup SSP AB, Sweden). Electrophoresis was performed at 90 mV for 1 h, after which the amplicons were visualised with the JY02G Fast Gel Imaging System (Beijing Junyi Dongfang Electrophoresis Co. Ltd., China). Preliminary *in silico* restriction site sequence analysis of the targeted CYTB gene (NCBI accession numbers: NC_000845.1 for pig, NC_001323.1 for chicken, NC_006853.1 for beef, and NC_001941.1 for sheep) was performed using Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/). Due to their assumed ability to discriminate between the species of interest and based on previously reported results (Ahmed & El-mezawy 2005; Farag *et al.* 2015), three enzymes were chosen for digestion: *Hae*III, *Rsa*I, and *Hinf*I. The expected restriction fragment lengths are listed in Table 1.

The obtained PCR products of the CYTB gene were digested using the three endonucleases (NEB, UK) in

Table 1. The hypothesised size of the fragment length polymorphisms obtained with three restriction endonuclease

Species	Restriction fragment length (bp)		
	*Hae*III	*Rsa*I	*Hinf*I
Pig (*Sus scrofa domestica*)	74, 132, 153	359	359
Chicken (*Gallus gallus*)	73, 127, 159	148, 211	10, 161, 188
Beef (*Bos taurus*)	74, 285	359	44, 117, 198
Sheep (*Ovis ovis*)	73, 127, 159	359	63, 296

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Table 2. Primer sequences and expected lengths of the PCR products (Doosti *et al.* 2014)

Species	Primer sequence	Product size (bp)	Annealing temperature (°C)
Chicken (*Gallus gallus*)	F: 5'-GGGACACCCTCCCCCTTAATGACA-3' R: 5'-GGAGGGCTGGAAGAAGGAGTG-3'	266	69
Beef (*Bos taurus*)	F: 5'-GCCATATACTCTCCTTGGTGACA-3' R: 5'-GTAGGCTTGGGAATAGTACGA-3'	271	63
Sheep (*Ovis ovis*)	F: 5'-ATGCTGTGGCTATTGTC-3' R: 5'-CCTAGGCATTTGCTTAATTTTA-3'	274	59

a total volume of 15 µl containing 12 µl PCR product, 10 × reaction buffer and 1 U of each restriction endonuclease. The reactions were incubated at 37°C for 3 h and resolved on 2.5% 0.5 × TBE agarose gels stained with GelRed™ Dropper (Olerup SSP AB, Sweden). The digests were electrophoresed at 60 mV for 2 h and photographed using the JY02G Fast Gel Imaging System (Beijing Junyi Dongfang Electrophoresis Co. Ltd., China). The restriction analyses were performed at least two times for each of the samples and each of the restriction enzymes.

For the purpose of obtaining specific segments of beef, chicken and sheep DNA, a set of species-specific primers previously reported by Doosti *et al.* (2014) were used. The primers together with the expected lengths of the amplified segments are presented in Table 2.

The SapphireAmp® Fast PCR Master Mix (Takara Bio Inc., Japan) was used for the PCR reactions as described for PCR-RFLP analysis. Cycling conditions consisted of 30 cycles of denaturation at 98°C for 10 s, annealing (63°C for beef, 69°C for chicken, and 59°C for sheep) and elongation at 72°C in a thermal cycler (Eppendorf Mastercycler Gradient; Eppendorf AG, Germany).

The obtained PCR products were visualised on a 1.5% 0.5 × TBE agarose gel stained with GelRed™ Dropper (Olerup SSP AB, Sweden) and photographed using the JY02G Fast Gel Imaging System (Beijing Junyi Dongfang Electrophoresis Co. Ltd., China).

RESULTS AND DISCUSSION

The 359-bp-long PCR fragment of CYTB was obtained for all animal species and from all investigated sampling regions of the sausages. As can be seen from Table 1, combining the use of three restriction enzymes should allow the detection of chicken, beef and sheep meat in heterogeneous pork products. As expected, in the sausages with added chicken meat, the *Hae*III enzyme did not generate a unique restriction profile, but unique restriction patterns were observed when using *Rsa*I and *Hinf*I enzymes (Figure 1) for all sampling regions and in all investigated amounts (i.e., 1 and 5% of added meat).

Unlike for chicken meat, we were not able to detect unique PCR-RFLP patterns either for beef or sheep DNA in pork/beef and pork/sheep mixtures. Moreover, the only PCR-RFLP pattern that could be detected was in sausages with added beef; the PCR products were digested with *Hae*III and this pattern was characteristic for pig (not shown).

In adulterated products, a small amount of foreign meat is usually added to the product to reduce production costs without disrupting sensorial properties. In the investigated sausages, there was obviously a much higher proportion of pork compared to beef and sheep meat (95 vs. 5 and 1%, respectively) and more pig DNA was amplified. Furthermore, in these kinds of mixtures a competition for primer binding sites can occur leading to preferential amplification of one species compared to the other species. In our case, pig DNA was amplified more efficiently, which could lead to non-detection of other animal species. This is in agreement with the results of Partis *et al.* (2000) and Maede (2006) who reported that, using *Hae*III and *Hinf*I, in most cases beef was not detected

Figure 1. The PCR-RFLP pattern obtained for the mixture of porcine and chicken meat

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when pork was present in meat mixtures. Moreover, Pietsch and Waiblinger (2010) reported that CYTB amplicons of the pig, turkey and chicken are amplified more efficiently than those of cattle and sheep, leading to reduced sensitivities in the mixture. According to the authors, the limit of detection (LOD) of beef in mixtures increases from 2% to around 20% in pork matrices. Partis *et al.* (2000) argued that this is a result of differential template amplification, where, depending on the extent to which different DNA templates are amplified, some tissues may go undetected even when present in high concentrations. Differential amplification generally results from primer mismatches that affect the stability of primer-DNA duplexes. To overcome this problem, Maede *et al.* (2006) proposed DNA sequencing of the obtained PCR products when by-products are visible in the PCR-RFLP profile, while Matsunaga *et al.* (1999) introduced a reaction with a common forward primer targeting a conserved region of the CYTB gene combined with species-specific reverse primers targeting the variable region of the same gene. The obtained results should then be verified using PCR-RFLP. In contrast to the results of this study, Partis *et al.* (2000) reported that as little as 10% lamb meat could be detected in mixtures using the above enzymes. However, the mixture in their investigation consisted of pork and sheep raw meat, which could lead to more efficient amplification of sheep DNA than in our study. Previous investigations have reported the efficient use of different restriction enzymes for differentiation of different animal species on the basis of the mitochondrial cytochrome b gene (Farag *et al.* 2015; Han *et al.* 2016), the COI gene (Haider *et al.* 2012; Spychaj *et al.* 2016), and 12s RNA (Girish *et al.* 2007; Chen *et al.* 2012). However, most of these investigations were conducted either on mixtures of raw meat, or on meat products which were not submitted to technological operations of smoking, salting, changes of pH etc., where much of the DNA is degraded, leading to a scenario in which the DNA of one species can be amplified more efficiently than that of the other.

Figure 2 shows the PCR products obtained using species-specific primers. PCR amplification revealed 271-, 274-, 149-, and 266-bp-sized fragments in the analysed mixtures.

The results of the species-specific PCR are in agreement with Doosti *et al.* (2014), who were able to detect all these species using specific primers in raw meat and different meat products. In recent years there have been a number of studies investigating the use of species-specific primers to discover adulteration in meat products. Tartaglia *et al.* (1998) reported a sensitivity range of 0.125–5% for detecting beef DNA in mixtures using L8129/H8357 specific primers, while Guoli *et al.* (1999) found a detection limit of 33.6 fg for raw beef samples. Kesmen *et al.* (2007) amplified different regions of mitochondrial genes using species-specific oligonucleotides for detection of horse, donkey, pig, beef and sheep DNA in cooked sausages and could detected the presence of as little as 0.01 ng DNA of each species. Furthermore, Karabasanavar *et al.* (2014) used species-specific primers targeting the porcine mitochondrial D-loop region and detected pork DNA in amounts of 10 pg. In our investigation, 1 and 5% meat were added to the prepared mixtures, and we were able to detect even as little as 1% of chicken, bovine and ovine DNA in the pork sausages.

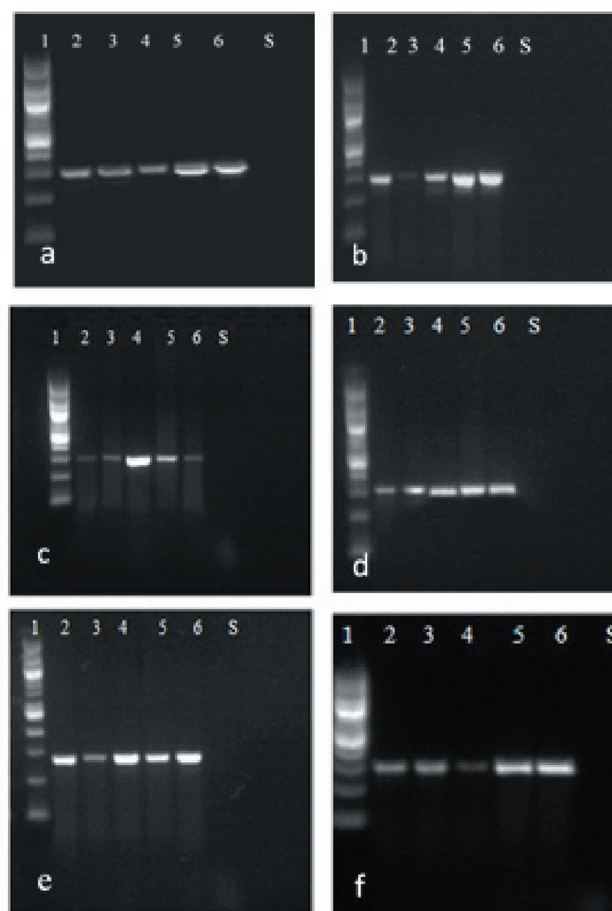

Figure 2. PCR fragments obtained using chicken-, beef- and sheep-specific primers for samples taken from five regions of pork sausages containing 1% and 5% chicken (a, b), beef (c, d), and sheep (e, f) meat

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CONCLUSIONS

The results of the present study show that PCR is indeed a powerful technique for the control of adulteration in meat products. However, the choice of method for detecting adulteration in fermented dry products is important as the DNA of one species can be amplified more efficiently than that of other species due to higher amounts of starting material present in the product or competition for primer binding sites. In these cases, it is more suitable to use species-specific primers: this method was proven to be both effective and sensitive enough for the discovery of even very low levels of adulteration in fermented dry meat products.

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