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Short Communication

Detection limit of polymerase chain reaction technique for species authentication in meat products

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Abstract

Authenticating a meat product according to the identity standard of the product and to its labeling means protect the consumers to a possible presence of meat from unknown and/or undesired species. This is a factor of paramount importance as it involves economic, public health and religious beliefs, such as in countries where the consumption of swine and its derivatives is forbidden. The aim of this study was to evaluate the detection limit of the PCR technique for the identification of swine and poultry species in meat products under different processing conditions. The research was developed based on mitochondrial DNA and oligonucleotide primers for amplification of 422 bp for poultry and 227 bp for swine. The sensitivity of the technique was evaluated with raw, cooked and autoclaved samples, which were intentionally adulterated at concentrations of 0%, 0.01%, 0.1%, 1%, 5% and 10%. A detection limit of up to 0.1% was found for samples of poultry adulterated with swine fat. However, the method was more accurate for samples with more than 5% intentional contamination. From the analyses that were performed it can be concluded that PCR is an effective tool that is sufficiently specific and sensitive for the identification of animal species in processed meat products, and it is therefore a viable alternative for routine analyses.

Introduction

The identification of animal species added to processed meat products is a constant concern, both for consumers and for the relevant authorities (Ballin, 2010). The aim is to authenticate the identity standard of the product linked to its correct labeling, thus protecting consumers from the possible presence of unknown and unwanted meat species (Arlan *et al*., 2006; Ghovvati *et al*., 2009; Soares *et al*., 2010).

The authenticity of meat products is of enormous importance because it involves economics, aspects of lifestyle, religion (e.g., prohibition of swine by Islam in Halal status and Judaism) and diet or health concerns (e.g., absence of allergens) (Calvo *et al*., 2001; Ballin *et al*., 2009; Ghovvati *et al*., 2009; Mane *et al*., 2009; Ballin, 2010; Nakyinsige *et al*., 2012). However, meat products are among the foods most subject to fraud due to difficulties in measurement and the economic benefits of processing, especially in the final product (Lago *et al*., 2011). Recently, issues related to the substitution of horsemeat for beef in fast food and ready-to-eat meals (burgers and lasagna) came to light in European countries (Che-Man *et al*., 2007).

Advances in molecular technology have enabled the development of alternative approaches to identify the protein in meat products, such as the application of polymerase chain reaction (PCR), which is considered reliable and effective because of its stability at high temperatures (Calvo *et al*., 2001; Kesmen *et al*., 2007; Bai *et al*., 2009; Ghovvati *et al*., 2009; Mane *et al*., 2009). This methodology, based on mitochondrial DNA (mtDNA), has the advantage of greater sensitivity and high specificity. This is because genes are present in thousands of copies per cell, and the large variability of mtDNA permits the reliable identification of specific species in mixtures as well as intraspecific variability with the possibility of identifying races (Arslan *et al*., 2006). The present study aimed to evaluate the detection limit of PCR for the identification of species of swine and poultry in meat products under different processing conditions.

Materials and Methods

In order to evaluate the sensitivity of the oligonucleotide primers to the presence of swine and poultry tissues, swine muscle (ham) and swine fat (lard) were added into poultry samples, as well as, poultry muscle (thigh and drumstick) and poultry fat were added into swine samples. These mixtures

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were prepared in concentrations of 0.01%, 0.1%, 1%, 5% and 10% for each species, in addition to a negative control sample for each species, for a total of 72 samples. The preparation was ground in a multiprocessor until it had the appearance of pâté. After that, 2% salt and 0.1% black pepper were added.

The samples were analyzed in the following processing conditions: raw; boiled in a water bath at atmospheric pressure until the center of the food reached 72°C and under pressure (autoclaved at 121°C for 10 minutes). After the heat treatment, the samples were stored frozen (-4°C).

The samples were first macerated and then submitted to the protocol of total DNA extraction described by Marcelino *et al*. (2008), with adaptations. After maceration, 500 µL of CTAB buffer (50 mM CTAB, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA and PVP 1%) was added to a 1.5 mL eppendorf tube, along with 100 mg of the respective sample. This was then mixed in a vortex for 5 minutes and then incubated in a dry water bath at 65°C for 30 minutes. For the deproteinization, 520 µL of CIA (24 parts chloroform: 1 part isoamyl alcohol) was added to the sample and then mixed in a vortex for 2 minutes and centrifuged at 12,000 rpm for 10 minutes. After centrifugation, the supernatant was transferred to a new 1.5 mL eppendorf tube, and a 1.0 volume of isopropanol and 0.5 volumes of 7.5 M of ammonium acetate were added. This was then centrifuged at 12,000 rpm for 10 minutes. The supernatant was then discarded, and the pellet was washed with 500 µL of refrigerated 70% ethanol. The mixture was then homogenized by inversion and centrifuged at 12,000 rpm for 5 minutes. Then the supernatant was discarded, the eppendorf tube was allowed to dry at room temperature for 15 minutes, and the content was then re-suspended in 50 µL of TE (1 mM EDTA and 10 mM Tris-HCl) and kept under refrigeration (-18°C).

The determination of the primers used in this study was based on aspects found in the literature. Primers and their main features are shown in Table 1. The reaction mixture was prepared in a 500 µL PCR tube with a total volume of 25 µL that contained the following: 10X PCR buffer (Invitrogen, Thermo Fisher Scientific Inc., USA); 200 µM of each dNTP (Fermentae, Thermo Fisher Scientific Inc., USA); 3.0 mM of $MgCl_2$ (Invitrogen, Thermo Fisher Scientific Inc., USA); 0.3 µM of each primer (Integrated DNA Technologies Inc., USA); 2.5 units of Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific Inc., USA); and 1 µg of target DNA and completed in volume with Milli-Q water. The PCR

Table 1. Sequence of primers used in the study of swine and poultry species

Species	Sense	Sequence (from 5' to 3')	Fragment size
Swine[1]	Sense	CATTCGCCTCACTCACATTAACC	227 bp
	Antisense	AAGAGAGAGTTCTACGGTCTGTAG	
Poultry[2]	Sense	CTCGCCCTACTTGCCTTCC	422 bp
	Antisense	TAGGACGCAACGCAGGTGT	

[1] Kesmen *et al*., 2007. [2] Mane *et al*., 2009.

conditions programmed into the Veriti thermocycler (Applied Biosystems Inc., USA) with 35 cycles as following: initial denaturation at 94°C for 3 minutes; denaturation at 94°C for 50 seconds; hybridization of primers at 70°C (swine) and 69°C (poultry) for 30 seconds; extension at 72°C for 1 minute; and final extension at 72°C for 10 minutes and 15°C until removal of sample equipment (Kesmen *et al*., 2007).

For verification of the results, an agarose gel (1.5%) electrophoresis system in a horizontal vat was used. After the gel was subjected to an electric current of 110V for 75 minutes, it was stained for 15 minutes with ethidium bromide (1 µg/mL). The results were then analyzed using a UV transilluminator and documented in a digital system. In the observation of the PCR product, the bands were compared with a standard molecular weight of 100 base pairs (bp). Positive and negative controls were conducted along with the samples as a control for the reaction.

Results and Discussion

Methods for the identification of the protein in foods of animal origin need to be accurate, authentic and rapid to protect the consumer from adulteration and to prevent unfair competition in the market (Ballin *et al.*, 2009). This is particularly relevant for populations whose religious beliefs forbid the ingestion of certain animal species, such as Jews and Muslims, who do not eat swine and its derivatives (Che-Man *et al*., 2007).

The pairs of primers selected for use in this study were based on the mitochondrial D-loop gene. Studies have found that it is possible to obtain more specific primers using mitochondrial DNA because mitochondrial DNA is inherited maternally. It means that there is only one allele in the individual, which removes ambiguity from the sequence (Kesmen *et al*., 2007; Mane *et al.*, 2009). In addition, the variable regions of mitochondrial DNA are present in thousands of copies per cell, increasing the probability of obtaining a positive result, even in the case of DNA fragmentation due to extreme

conditions during processing, such as the use of high temperatures. This is a feature that makes it ideal for the identification of animal species in processed meat products (Arslan *et al*., 2006).

The PCR assay was evaluated for its detection limit in the amplification of DNA extracted from poultry meat that was intentionally adulterated with swine meat and swine fat. In addition to testing its effectiveness with different heat treatments (raw, cooked and autoclaved meat), the methodology was effective in amplifying fragments of 442 bp of DNA extracted from poultry.

No adverse effects were observed among the different thermal treatments because amplification occurred in all the samples containing 10% adulteration with swine (227 bp) (Table 2). A similar result was found by Ulca *et al.* (2013), who evaluated PCR in real time by analyzing raw meat and meat products cooked for 20 minutes at 200°C; they obtained amplification for 0.1% of swine, independent of the processing that was used.

Analyzing the detection limit for the adulteration of poultry meat with swine fat, the methodology showed a high degree of sensitivity because there was amplification from the concentration of 0.1% in the raw samples. For the samples that were heat treated, a band of 5 to 10% was obtained, even after the samples underwent autoclaving at 121°C for 10 minutes. As previously mentioned, this result may be due to the large number of copies of mitochondrial DNA in the tissues, thus contributing to the survival of a sufficient number of copies of DNA, as well as stability to heat, even when subjected to extreme conditions (Mane *et al*., 2009).

The replacement of one animal species with another that is economically cheaper, or the labeling of a product that is not consistent with its identity, may affect thousands of consumers who rely on quality and transparency regarding products. Fraudulent behavior in this respect is widespread because meat content is not easily measured, which has led to an increase in research aimed at enhancing methodologies capable of identifying the constituents of processed meat products. In this context, the use of mitochondrial DNA and the primers used in this phase of the research were satisfactory, which testifies to the high sensitivity of the method.

Kesmen *et al.* (2007) also successfully used PCR assays for the identification of animal species present in cooked poultry sausages. As for the sensitivity of their assays, amplification was detected in mixtures with less than 1% adulteration without any adverse effects of the processing conditions (heat) or the ingredients used in the preparation of the sausages.

Table 2. Results of sensitivity testing of the PCR technique for different concentrations and different heat treatments of swine and poultry species

Meat Processing	Adulterating Level (%)	In Poultry Meat		In Swine Meat	
		Swine Meat Detection*	Swine Fat Detection	Poultry Meat Detection	Poultry Fat Detection
Raw	0	-	-	-	-
	0.01	-	-	-	-
	0.1	-	+	-	-
	1	-	+	-	-
	5	+	+	+	-
	10	+	+	+	-
Cooked under pressure	0	-	-	-	-
	0.01	-	-	-	-
	0.1	-	-	-	-
	1	-	-	-	-
	5	-	+	+	-
	10	+	+	+	-
Cooked under pressure ATM	0	-	-	-	-
	0.01	-	-	-	-
	0.1	-	-	-	-
	1	-	-	-	-
	5	-	+	+	-
	10	+	+	+	+

* Results expressed with '+' means that the adulterating was detected as well as results with '-' means that the adulterating could not be detected.

The PCR assay was also analyzed for its detection limit in the amplification of the DNA extracted from swine that was intentionally adulterated with poultry meat and poultry fat. The PCR assay was suitable for the amplification of the swine species, obtaining a band with 227 bp as described by Kesmen *et al.* (2007).

The amplification was positive in all the samples of swine meat, without showing adverse effects regarding processing conditions or the ingredients used to prepare the samples. This result was also observed in a study by Calvo *et al.* (2002), which successfully developed primers specifically for the identification of swine in meat products such as sausages, burgers and pâté.

In the present study, PCR detected adulteration in the samples containing 5 and 10% poultry meat, regardless of the heat treatment that was used. These results make it clear that the efficiency of the technique is not affected by the use of heat on the sample, making it ideal for identifying the protein in processed meat products (Mane *et al*., 2009). These results were not repeated when analyzing the swine sample that was adulterated with poultry fat. Even though the primer for swine species showed positive results for all the samples with no change in samples that underwent thermal treatment the limit of detection (sensitivity) of the initiator of the poultry species was unsatisfactory, amplifying only

one sample.

The amplification only in the sample with the highest concentration of adulteration (10%) and milder heat treatment (cooking in a water bath at 72°C) may result from the difficulty of extracting DNA that was observed in this study. Foods with a high fat content hamper the extraction of cleaner DNA and may also contain contaminants and active enzymes that can inhibit the reaction and thereby compromise the results as compared with the use of simple raw material.

The samples highlighted in yellow indicate positive results for the detection of adulteration. The results indicated that adulterating with the inclusion levels lower than 5% in most cases could not be detected by PCR method, regardless of original meat species or the product (meat or fat) used in the adulterating.

The PCR method was successful for the detection of adulteration by swine and poultry in meat products, with sensitivity similar to tests conducted by Arslan *et al*. (2006), Che-Man *et al*. (2007) and Haunshi *et al*. (2009). These results indicate that PCR is a rapid, specific technique that is able to detect adulteration in meat products subjected to different heat treatments.

Conclusion

By using PCR, it was possible to identify the addition of fat swine in poultry products from 0.1%. However, the method was more accurate for samples with more than 5% intentional contamination. It was also possible to detect the addition of poultry to swine products. The thermal treatments did not influence the level of detection; consequently, PCR can be regarded as a robust method, even when using more invasive thermal treatment procedures such as autoclaving. It is possible to use the PCR technique for routine analysis to identify the species of animal used in meat products in order to protect consumers against fraudulent practices such as the replacement of meat. It is also an alternative method of establishing the authenticity of the identity pattern of the final product for industrial purposes.

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References

Arslan, A, Ilhak, O. I. and Calicioglu, M. 2006. Effect of method of cooking on identification of heat processed beef using polymerase chain reaction (PCR) technique. Meat Science 2: 326-330.

Bai, W., Xu, W., Huang, K., Yuan, Y., Cao, S. and Luo, Y. 2009. A novel common primer multiplex PCR (CP-M-PCR) method for the simultaneous detection of meat species. Food Control 4: 366-370.

Ballin, N. Z. 2010. Authentication of meat and meat products. Meat Science 3: 577-587.

Ballin, N. Z., Vogensen F. K. and Karlsson, A. H. 2009. Species determination: can we detect and quantify meat adulteration? Meat Science 2: 165-174.

Calvo, J. H., Zaragoza, P. and Osta, R. 2001. Technical note: a quick and more sensitive method to identify pork in processed and unprocessed food by PCR amplification of a new specific DNA fragment. Journal of Animal Science 8: 2108-2112.

Calvo, J. H., Rodellar, C., Zaragoza, P. and Osta, R. 2002. Beef- and bovine-derived material identification in processed and unprocessed food and feed by PCR amplification. Journal of Agricultural and Food Chemistry 19: 5262-5264.

Che-Man, Y. B., Aida, A. A. A., Raha, A. R. and Son, R. 2007. Identification of pork derivatives in food products by species-specific polymerase chain reaction (PCR) for Halal verification. Food Control 8: 885-889.

Ghovvati, S., Nassiri, M. R., Mirhoseini, S. Z., Moussavi, A. H. and Javadmanesh, A. 2009. Fraud identification in industrial meat products by multiplex PCR assay. Food Control 8: 696-699.

Haunshi, S., Basumatary, R., Girish, P. S., Doley, S., Bardoloi, R. K. and Kumar, A. 2009. Identification of chicken, duck, pigeon and pig meat by species-specific markers of mitochondrial origin. Meat Science 3: 454-459.

Kesmen, Z., Sahin, F. and Yetim, H. 2007. PCR assay for the identification of animal species in cooked sausages. Meat Science 4: 649-653.

Lago, F. C., Herrero, B., Madriñán, M., Vieites, J. M. and Espiñeira, M. 2011. Authentication of species in meat products by genetic techniques. European Food Research and Technology 232: 509-515.

Mane, B. G., Mendiratta, S. K. and Tiwari, A. K. 2009. Polymerase chain reaction assay for identification of chicken in meat and meat products. Food Chemistry 3: 806-810.

Marcelino, F. C., Guimarães, M. F. M. and Barros, E. G. 2008. Detection and quantification of Roundup Ready soybean residues in sausage samples by conventional and real-time PCR. Food Science and Technology 28: 38-45.

Nakyinsige, K., Man, Y. B. C. and Sazili, A. Q. 2012. Halal authenticity issues in meat and meat products. Meat Science 3: 207-214.

Soares, S., Amaral, J. F., Mafra, I. and Oliveira, M. B. 2010. Quantitative detection of poultry meat adulteration with pork by a duplex PCR assay. Meat Science 3: 531-536.

Ulca, P., Balta, H., Çagin, I. and Senyuva, H. Z. 2013. Meat species identification and Halal authentication using PCR analysis of raw and cooked traditional Turkish foods. Meat Science 3: 280-284.