MULTIPLEX PCR ASSAY FOR DETECTION AND IDENTIFICATION OF ANIMAL SPECIES IN THE MEAT PRODUCTS

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Abstract

Meat products and by-products adulteration is a real food safety problem that interests equally consumers and the authorities. The existence of specific genetic variation on mtDNA make it possible to detect and identify the animal meat species from fresh meat products or processed food products. In the present work we developed a multiplex PCR assay for identification of some animal species: mammals (cattle and pork), birds (poultry) and fish, from market meat products. Each primer pair was tested individual regarding specificity to a mitochondrial genome but also in a DNA mixture of all four analysed species. The specific hybridization of each primer pair has led to the production of characteristic size PCR products of 374 bp for beef, 290 bp for pork, 224 bp for poultry and 181 bp for fish. The multiplex protocol was then tested for species-specific identification in different commercial meat by-products.

Key words: mtDNA, meat, species identification, Multiplex PCR

INTRODUCTION

In the theory of the evolution of species, the DNA molecule appears to have changed during the evolution, and these changes (though most neutral) allow the identification of species from different biological sources. These modifications (even majority neutral) allow species identification from various biological sources. The level of intraspecific diversity must be confirmed before assuming that certain sequences can be discriminative. It is possible today to evidence these variations because more and more genome species are sequenced and annotated and can be accessed in the genetic databases. Finding the species - specific variations that can be amplified simultaneously in a single PCR reaction (multiplex) can be difficult, but it can reduce considerably the costs in a laboratory to be applied with accuracy in a single assay. The accelerated evolution rate in the mtDNA denotes the possibility to find significant amounts of sequence variations in closely related species [15]. Adulteration of meat products and by-products remains a problem in all countries, and a matter of equal concern for consumers and authorities, on account of safety and health security of the population. In this regard, after the outbreak of scandal related to horse meat added as a substituent for beef, several legislative requirements were formulated. “European food labelling legislation Regulation (EU) No. 1169/2011 on Food Information to Consumers introduced in December 2014 sets out requirements for “voluntary labelling” – including country of origin – stating that any additional voluntary claims must not mislead, be ambiguous or confuse consumers [18].” On 13 March 2018, the European Commission is launching a Knowledge Centre for Food Fraud and Quality, operated by the Joint Research Centre [19]. In this view, the first analytical methods based on identification of proteins sensitive to thermic treatment were replaced with DNA based methods. Depending on implementing costs, available technology, accuracy and complexity of the requirements for species identification, it can be classified in three major groups: (1) based on PCR amplification [3; 5; 7; 8; 10; 11; 13; 15; 16; 17], (2) hybridization [4; 6; 9; 12] and (3) sequencing [2;14;1]. he aim of this study was the development of a simple method based on PCR amplification for detection and rapid

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identification of the most common animal species present in the commercial products, either as raw meat or processed one. The method does not propose to identify the particularity of taxonomic grouping of the species being tested for rapid quality identification of beef, pork, poultry and fish products.

MATERIAL AND METHOD

***Samples collection*:** Two experiments were carried out to detect and identify the animal species from fresh meat and processed meat by-products. The first experiment was set to test cross-hybridization of primer pairs. For the subsequent PCR multiplex analysis of this experiment, DNA samples of all four species were used separately or in pool. The first four PCR mixtures included all primer pairs and an individual DNA of one animal species and the following samples included DNA mixture in pool: a) one pool with 1 µl DNA from beef and poultry, b) one pool with 1 µl of each DNA: beef, pork, poultry and fish; c) one pool with 1 µl of each DNA: beef, poultry and fish; d) one pool with 1 µl of each DNA: pork and poultry; e) one pool with 1 µl of each DNA: beef, pork and fish; f) one pool with beef, pork and poultry DNA g) one pool with poultry and fish DNA. The second experiment was conducted with DNA from samples of commercial meat products.

DNA isolation: For Multiplex PCR in the first experiment DNA was extracted from fresh meat (raw samples of beef, pork, poultry and salmon), and in the second experiment from different labelled or unlabelled processed food: sausages, wiener sausages, hamburgers, fish fingers, mixed meat (poultry, beef and pork, fish mixture, etc). The DNA was isolated with Quick DNA Microprep Plus Kit following the manufacturer's instructions (BioZyme). The DNA samples were analysed to determine the concentration and purity with Nano Drop ND 1000. All samples had the optimum purity ranging between 1.8-2 and a quantity of 44 -100 ng/µl.

***Primer design*:** The multiplex PCR allows to simultaneously amplify different DNA regions in a single assay. To meet the specific requirement of cyclic amplification of DNA regions in three steps in a PCR reaction: denaturation, annealing and elongation, the only one step, that of annealing the primer to the template, must be at the same temperature for all primer pairs. This makes it difficult to set the primer pair's structure with all requirements needed to make a proper design. The primer design was made using the Primer 3 Premier software for pork and poultry species, and for beef and fish, the primers structure, described previously by other authors, was verified with Primer Blast tool, available on NCBI. The accession number on NCBI for mitochondrial genomes of all analysed species, the primer pair's structure, the genomic position and the genes to be amplified are presented in Table 1.

Table 1 The Primer's structure for specific identification of each species in Multiplex PCR, Genebank mitochondrial accession number, primer hybridization position on the genome and genes to be amplified on each genome species

Accession number	Species	Primer structure	Genomic positions/genes	References
EU177870.1	*Bos taurus*	5' GAAAGGACAAGAGAAATAAGG 3' 5' TAGCGGGTCGTAGTGGTTCT 3'	2291-3294 16S rRNA, Leu tRNA, ND1	[15]
NC_000845	*Sus scrofa*	5' CTACATGAGTATATCCACCACA 3' 5' ACATTGTGGGATCTTCTAGGT 3'	1992 – 2260/ 12S rRNA, tRNA-Val	In this work
NC_040902.1	*Gallus gallus*	5' ACCCGGCGTAAAGAGTGG 3' 5'TAGGGCTAGGCATAGTGGGG 3'	1587-1768 12S rRNA	In this work
NC_002616.1	*Sardinops melanostrichus*	5' TAAGAGGGCCGGTAAAACTC 3' 5' GTGGGGTATCTAATCCCAG 3'	291-514 12 rRNA	[5]

Multiplex PCR:

1. The test for cross-hybridization of primer pairs.

The multiplex PCR reactions were set to a final volume of 35 µl with different DNA quantity and all primer pairs for the first experiment (Table 2). The composition of PCR mix was as follows: 7 µl **5** x Firepol Ready to load with 12.5 Mm $MgCl_2$ (ByoZime), 2 µl primer (10pmol/µl solution of each, forward and reverse), 1 µl DNA template for each species, and ultrapure H_2O to a final volume of 35 µl.

2. The multiplex PCR assays for detection and identification of animal species in commercial meat products and by-products were conducted in 25 µl final volume with: 5 µl 5 x Firepol Ready to load with 12.5 Mm $MgCl_2$ (ByoZime), 2 µl primer (10pmol/µl solution of each, forward and reverse), 2 µl DNA template for each commercial product (table 3), and 16 µl ultrapure H_2O. Amplification was performed in an Eppendorf Master Cycler thermocycler (Eppendorf, Germany) with the following cycling conditions : 94°C (5 min) – 1 cycle; 34 cycles x 94°C – (30 s); 60°C (30 s) and 72°C (45 s); and final extension at 72°C for 8 min, maintaining 4°C thereafter.

Table 2 Species composition used in first experiment for testing the cross-hybridization of primer pairs

Samples	DNA pool	Species	Primer pairs used
1	-	Bovine	All
2	-	Pig	All
3	-	Chicken	All
4	-	Salmon	All
5	a)	Bovine + chicken	All
6	b)	Bovine + Pig + chicken+salmon	All
7	c)	Bovine + chicken+salmon	All
8	d)	Pork+ chicken	All
9	e)	Bovine + Pig + salmon	All
10	f)	Bovine + Pig + chicken	All
11	g)	chicken+salmon	All

Table 3 Species composition used in the second experiment for detecting and identifying the animal species in commercial products

No	Sample	Labelled species
1	Minced beef	Bovine
2	Chicken burger	Chicken
3	Chicken sausages	Chicken
4	Fish fingers	Fish (*Gadus morhua*)
5	Frozen fish	Salmon
6	Beef burger	Bovine
7	Beef meal	Bovine
8	Beef and pork lasagna	Bovine and Pig
9	Beef meal	Bovine
10	Beef and chicken meal	Bovine and chicken
11	Minced meat meal	Bovine+ Pig
12	Pork sausages	Pork
13	Pork sausages	Pork
14	Beef meal	Bovine
15	Minced beef and pork meat	Bovine+ Pig
16	Beef burger	Bovine
17	Chicken meal	Chicken
18	Frozen fish Filet	Fish (*Sardina* sp)
19	-	-
20	Minced Pork meal	Pig

Electrophoresis of PCR products:

Amplimers were submitted to electrophoresis in a 2.5 % agarose gel, with Tris - Borate - EDTA buffer 1 x for 1oo minutes at 90 V and stained with SybrSafe 1 µl/10 ml medium.

RESULTS AND DISCUSSION

In the first experiment amplification was conducted with DNA from raw materials (beef, pork, chicken and salmon) and all primer pairs to verify the specificity of primers. For this purpose, each DNA sample was amplified with all primer pairs, resulting specific fragments of 374 bp for beef, 290 bp for pork, 181 bp for chicken and 224 bp for salmon. The second step was to test the cross-hybridization of primer pairs to different DNA. In this purpose different DNA pools were submitted to amplification multiplex with all primer pairs. The DNA species from each pool are presented in table 2. The results of the first experiment indicate the species-specific hybridisation of each primer pair and a characteristic size PCR product for each animal species (fig. 1).

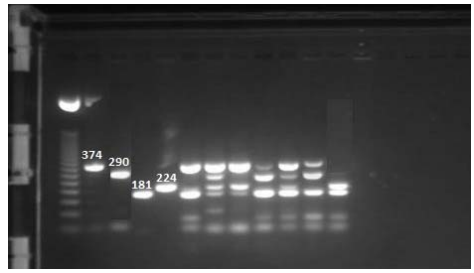

Fig. 1 Electrophoretic profiles in the first experiment of Multiplex PCR reactions. Line 1/50 bp DNA marker, line 2 - 374 bp for beef sample, line 3 / 290 bp for pork DNA sample, line 4/181 bp for poultry DNA and line 5/224 bp for salmon DNA; line 6/ DNA pool a); line 7/DNA pool b); line 8/DNA pool c); line 9/DNA pool d); line 10/DNA pool e; line 11/DNA pool f; line 12/DNA pool g.

The second experiment tested the primers' specificity to detect and identify animal species in different processed commercial products. The meat products were purchased from different places such are supermarket, fast food, restaurants. The composition of the products (labelled or unlabelled) was taken from labels or from the menu. The amplification of the DNA extracted from food samples led to different profiles in the agarose gel electrophoresis, with a band of different size corresponding to a species – specific profile. These sizes are the same as in the first experiment, with 374 bp for beef, 290 for pork, 181 for chicken and 224 for fish. The samples used in the experiment are presented in table 3 and the multiplex PCR profiles in figure 2.

The results of Multiplex PCR performed on commercial meat products revealed the presence of ten wrongly labelled products on the market (Table 4). For some products it was specified on the label that they could contain traces of meat from other species.

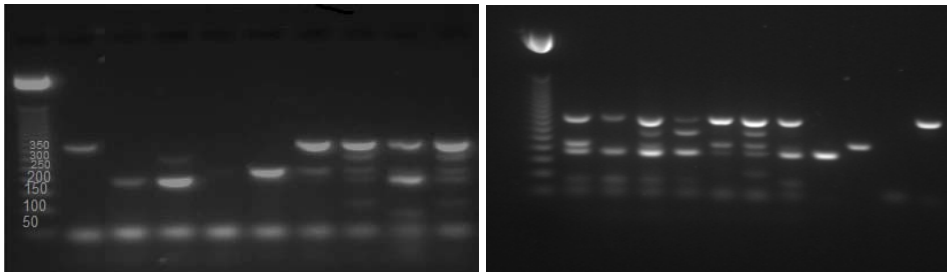

Fig. 2 (a) Line 1 – 50 bp DNA marker, line 2/ Minced beef; line 3/ Chicken burger, line 4/ Chicken sausages, line 5/ Fish fingers, line 6/ Frozen fish; line 7/ Beef burger, line 8/ Beef meal; line 9/ Beef and pork lasagne. Fig 2 (b).Line 1 – 50 bp DNA marker; line 2/ Beef meal; line3/ Beef and chicken meal; line 4/minced meat meal; line 5/ Pork sausages; line 6/ Beef meal; line 7/ minced beef and pork; line 8/ Beef burger; line 9/ Chicken meal; line 10/ Frozen fish Filet; line 11/-; line 12/minced pork meal

Table 4 Results of PCR multiplex performed on commercial products

No	Sample	Labelled species	Results
1	Ground beef	Bovine	Beef
2	Chicken burger	Chicken	Poultry
3	Chicken sausages	Chicken	Pork and Poultry
4	Fish fingers	Fish (*Gadus morhua*)	-
5	Frozen fish	Salmon	Fish
6	Beef burger	Bovine	Beef+Fish
7	Beef meal	Bovine	Beef + Pork+ Poultry +fish
8	Beef and pork lasagna	Bovine and Pig	Beef + Poultry
9	Beef meal	Bovine	Beef
10	Beef and chicken meal	Bovine and chicken	Beef + Poultry + fish
11	Minced meat meal	Bovine + Pig	Beef + Poultry
12	Pork sausages	Pig	Beef+ Poultry +pork
13	Pork sausages	Pig	Pork + Poultry
14	Beef meal	Bovine	Beef + fish
15	Minced beef and pork meat	Bovine+ Pig	Bovine + pork+ Poultry +fish
16	Beef burger	Bovine	Beef + Poultry
17	Chicken meal	Chicken	Poultry
18	Frozen fish Filet	Fish (Sardina sp)	Fish
19	-	-	-
20	Minced Pork meal	Pig	Pork

CONCLUSIONS

As was demonstrated in the first experiment, the primer pairs evidenced species-specificity to a certain mitochondrial genome. This fact was previously tested in silico, in the BLAST analysis. The specificity of primers for cattle is to amplify the genomic region which includes the following genes: 16S rRNA, tRNA- Leu, and ND1, while the primers for pork were designed to amplify genes codifying 12S rRNA and tRNA-Val. For chicken genome, the primers were designed to amplify a region from 12S rRNA, the same gene as for Sardinops sp. being identified the specificity of each primer pairs for each genome, with a particular size of PCR amplimers. It should be noted that fish primers did not align with the poullach genome (*Gadus morhua*) but they aligned with the sardine and salmon genome. The other fish species that can be identified with this primers are described by Dalmasso et al., (2004). The method proves to be reliable for rapid animal species identification in a multiplex PCR assay.

REFERENCES

[1]. Ballin, N.Z. (2010) Authentication of meat and meat products. *Meat Sci* 86:577–587.

[2]. Bertolini, F., Ghionda, M.C., D'Alessandro, E., Geraci, C., Chiofalo, V., Fontanesi, L. (2015) A Next Generation Semiconductor Based Sequencing Approach for the Identification of Meat Species in DNA Mixtures. *PLoS ONE* 10(4): e0121701

[3]. Cai, Y., He, Y., Lv, R., Chen,H., Wang,H., Pan, L. 2017. Detection and quantification of beef and pork materials in meat products by duplex droplet digital PCR. *PLoS One.* 3;12(8):e0181949.

[4]. Chikuni, K., Ozutsumi, K., Koishikawa, T. and Kato, S. (1990) . Species Identification of Cooked Meats by DNA Hybridization Assay. *Meat Sci*. 27, 119±128

[5]. Dalmassoa, A., Fontanellab, E., Piattib, P., Civeraa, T., Rosatic, S., Botteroa, M.T. (2004) A multiplex PCR assay for the identification of animal species in feedstuffs. *Molec and Cell Probes*. 18Ş 81–87

[6]. Ebbehuj, K.F. , Thomsen, P.B. (1991) Species Diferentiation of Heated Meat Products by DNA hybridization. *Meat Sci.* ;30(3):221-234.

[7]. Ghovvati, S., Nassiri, M. R., Mirhoseini, S. Z., Moussavi, A. H., & Javadmanesh, A. (2009). Fraud identification in industrial meat products by multiplex PCR assay. *Food Control*, 20,696–699.

[8]. Girish, P. S., Anjaneyulu, A. S. R., Viswas, K. N., Anand, M., Rajkumar, N., Shivakumar, B. M., (2004). Sequence analysis of mitochondrial 12S rRNA gene can identify meat species. *Meat Sci,* 66, 551–556
[9]. Hunt, D.F., Parkes, H.C., Lumley, L.D., (1997). Identification of the species of origin of raw and cooked meat products using oligonucleotide probes. *Food Chem*. 60,437- 442
[10]. Ivanova, N.V. , Zemlak, T.S., Hanner, R.H., Hebert, P.D.N. (2007) Universal primer cocktails for fish DNA barcoding. *Mol Ecol Notes* 7:544–548.
[11]. Lockley A.K., Bardsley R.G., 2000. DNA-based methods for food authentication. *Food Sci and Technol.* 11: 67±77
[12]. Matsunaga, T., Sato, T. and Tsuji, K. (1998). Diferentiation of Japanese Black Cattle and Other Cattle Breeds by DNA Hybridisation. *J. Jap. Soc. Food Sci. Tech*. 45, 155±157
[13]. Perreira, F., Carneiro, J., Amorim, A. (2008). Identification of Species with DNA-Based Technology: Current Progress and Challenges. *Recent Patents on DNA & Gene Seq*, 2, 187-200
[14]. Rodríguez-Ramírez, R., González-Córdova, A.F., Vallejo-Cordoba,B. (2011) Review: Authentication and traceability of foods from animal origin by polymerase chain reaction- based capillary electrophoresis. *Anal Chim Acta* 685:120–126
[15]. Sakalar, E., Abasiyanik, M.F. (2011). Qualitative analysis of meat and meat products by multiplex polymerase chain reaction (PCR) technique. *African J of Biotechnol*. 10 (46), 9379-9386
[16]. Unseld, M., Beyermann, B., Brandt, P. and Hiesel, R. (1995) Identification of the Species of Origin of Highly Processed Meat Products by Mitochondrial DNA Sequences. *PCR Meth. App*. 4, 241-243
[17]. Walker, M.J., Burns, M., Burns, T.J, Horse Meat in Beef Products- Species Substitution 2013, J of the Assoc of Public Anal (Online) 41: 67-106
[18]***https://eur-lex.europa.eu/legal-content/RO/TXT/PDF/?uri=CELEX:32011R1169&from=EN.
[19].***https://ec.europa.eu/jrc/en/news/commission-launches-knowledge-centre-boost-food-quality-and-step-fight-against-food-fraud