Bulletin UASVM Animal Science and Biotechnologies, 68(1-2)/2011
Print ISSN 1843-5262; Electronic ISSN 1843-536X

Applicability of a Multiplex PCR Assay for the Identification of Animal Species in Feedstuffs

Oana M. IOJA-BOLDURA[1)], Sorina POPESCU[1)], Silvia BRUZNICAN[1)], Ioan HUTU[2)]

[1)]Banat University of Agricultural Sciences and Veterinary Medicine,
Molecular Genetic Laboratory, Timişoara, Romania
[2)]Extension Unit of Banat University of Agricultural Sciences and Veterinary Medicine,
119 Aradului Road, 300645 Timişoara, Romania; oanaboldura@gmail.com

Abstract. The identification of animal species used in industrial meat products is very important, from economical considerations, in European Union, which has implemented a set of very strict procedures to correctly label food. In this paper we analyzed conserved region from mitochondrial 12S rRNA and 16S rRNA genes, a powerful region to evaluate the presence of fraudulently added meat in compound food.

The multiplex PCR proposed in this study can be considered a valid alternative to the microscopic method for the detection of animal derived materials banned by an European Union Regulation. This assay can be used for the identification of most species (cattle, poultry, swine and fish) fulfilling the need for a high sensitivity method to assess food authentication.

Keywords: meat products; species identification; multiplex PCR

INTRODUCTION

Food authenticity is currently an issue of major concern for food authorities, since incorrect labeling of animal food may have negative consequences. The application of quality assurance systems through the food chain requires the development of reliable and simple tools, which facilitate routine control assessments. Incorrect labeling represents commercial fraud as regards the consumer (Ghovvati *et al.,* 2009) and may also have implications for health, especially in case of consumers who exhibit sensitivity to undeclared antigens. To overcome these problems, molecular methods of identification have been developed, based on analysis of either protein or DNA. Reliable techniques to identify the origin of derived product's components regarding the species are necessary for food authentication purpose (Bottero *et al,* 2003). In the last two decades, considerable efforts have been invested in the development of DNA based speciation. This was done in the belief that short nucleic acid sequences are able to survive food processing much better than the protein epitopes that are currently the basis of immunochemical methods. Furthermore, the ability of PCR to amplify small amounts of specific DNA target sequences has considerable advantage over the most methods for polypeptide identification.

Meat proteins and DNA molecules have been used as species-specific biological markers for meat species identification. Methods that use protein analysis include electrophoretic, immunological, mass-spectrometric and chromatographic techniques (Nierderer and Bollhalder, 2001). However, proteins lose their biological activity after an animal dies, and their presence and characteristics depend on cell types and thus, processing can alter the structure and stability of meat. Furthermore, most of them are heat degradable.

Thus, for meat species identification, DNA analysis would be preferable to protein analysis. The identification in cooked meat is difficult, since the temperatures during heat treatment destroy species-specific proteins or their epitopes (Gouli, *et al.*, 1999). Alternatively, DNA analysis constitutes an attractive strategy for meat species identification. In comparison with proteins, DNA is stable against technological treatments and independent of the considered tissue.

PCR analysis of species-specific mt DNA sequences is the most common method currently used for species identification (Parodi *et al.,* 2002). Detection method based on mt DNA can improve the assay sensitivity because each cell has only a set of genomic DNA in the nucleus, but bearing several copies of mtDNA. The mtDNA has a high mutation rate, poor corrective replication of polymerase and lack of proof-reading system in the organelle. Since mt DNA expressed in different species or genuses have their evolution specificities, we can identify individual species by studying mt DNA.

MATERIALS AND METHODS

Beef, pork, chicken and fish raw meat and maize seeds and eight types of food products: four commercial meat products and four pet foods were analyzed (Tab. 1). The specificity sample, consisted of a DNA mix of the four species (cattle, swine, poultry and fish), was diluted in vegetable DNA (maize) up to 0.001%. The commercial meat products were obtained from the retail trade.

The samples were frozen and manually grinded. The maize seeds materials were mill grinded. The products were homogenized and then the analytical samples were prepared. For each matrix, sample of 100 mg were used in the DNA extraction.

Tab. 1

Samples submitted to assay

Samples	Species
Beef	*Bos taurus*
Pork	*Sus scrofa*
Poultry meat	*Gallus gallus domesticus*
Fish meat	*Merlucius vulgaris*
Sausages (A)	Chicken
Processed meat product (B)	Pork
Processed meat product (C)	Pork/ others
Raw meat product (D)	Pork/bovine
Pet food (E)	Beef/turkey
Pet food (F)	Poultry/lamb
Pet food (G)	Poultry/others
Pet food (H)	Fish/others
Maize	Maize flour

DNA extraction and purification method

The DNA was extracted using CTAB method following the standard procedure validated in our laboratory. The method is used for the extraction and purification of DNA from a wide range of raw and processed matrices, being particularly suitable for the elimination of polysaccharides and polyphenolic compounds, otherwise affecting the DNA purity and therefore quality (Querci *et al.,* 2004). Each sample was extracted in two copies; in addition, an extraction blank control (EB) and an environment control (EC) were done.

Quantification of the extracted DNA by spectrophotometric method

The quality and quantity of extracted DNA was assessed by spectrophotometry method (BioMate Spectrophotometer-ThermoScientific). DNA was evaluated directly in aqueous diluted solution, measuring the optical density (OD) in ultraviolet light. The concentration of nucleic acids was determined by measuring at 260 nm against a blank. The quality of extracted DNA was evaluated by A_{260}/A_{280} ratio.

PCR analyzes

Four sets of primer were used in this study for PCR amplification (Tab. 2). Species-specific primers were designed from different regions of mitochondrial DNA (12S rRNA,tRNA Val and 16S rRNA). These primers were published by Dalmasso *et al.* (2004). The primers were synthesized by *Eurogentec S.A., Belgium.*

Tab. 2

The primer sequences for the different animal species

Species	Genes	Primers 5'-3'		Amplicons (bp)
cattle	16S rRNA	TAA GAG GGC CGG TAA AAC TC	GTG GGG TAT CTA ATC CCA G	104
swine	12S rRNA-tRNA	CTA CAT AAG AAT ATC CAC CAC A	ACA TTG TGG GAT CTT CTA GGT	290
poultry	12S rRNA	TGA GAA CTA CGA GCA CAA AC	GGG CTA TTG AGC TCA CTG TT	183
fish	12S rRNA	TAA GAG GGC CGG TAA AAC TC	GTG GGG TAT CTA ATC CCA G	224

For the separate detection of animal species in the samples they were subjected to analyses. PCR amplification was performed in a final volume of 25 µl using Go Taq Green Master Mix PCR kit from Promega, 20 pmol of primers and DNA template.

For the simultaneous detection of each species, one step multiplex PCR was developed using each of the primer sets previously designed for the simplex PCR. The same master mix as described for simplex PCR was used, primers concentrations being as follows: 20, 20, 12.5 and 10 pmol of beef, pork, fish and poultry primers.

The choice of template concentration depends on the sample nature: for raw meat sample 200 ng of DNA template was used, in the case of feedstuffs, where a low concentration of animal DNA is expected, 600 ng were used.

Amplification was performed in a *Corbett RESEARCH* Thermal Cycler with the following cycling conditions; after an initial heat denaturation at 94 °C for 10 min, 35 cycles were programmed as follows: 94°C for 30 s, 60°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min.

Amplicons were analyzed by 3% agarose gel electrophoresis (Promega, USA) run in Tris Acetate EDTA Buffer for 30 min at 95 V and visualized in Ethidium Bromide (0.4 ng/ml) presence.

RESULTS AND DISSCUSION

In a preliminary phase of this investigation multiplex PCR specificity was assessed with DNA extracted from raw meat along with the sample consisted of the mixture of all four species DNA. The primers generated specific fragments of 104 bp for ruminants, 183 bp for poultry, 225 bp for fishes and 290 bp for pork. When multiplex PCR was carried out on

described samples, the set of primers retained the same specificity; the result may be seen in *Figure 1*. The electrophoretic pattern clearly shows the absence of cross-reaction. In fact, only the specific species band is evident.

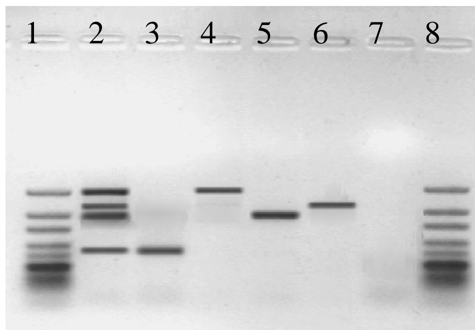

Fig. 1. Multiplex PCR specificity for DNA extracted from different types of raw meat: lane 1, molecular weight marker: UltraLow Range DNA Ladder, Fermentas. lane 2, mix of ruminant, poultry fish and pork; fish; lane 3, ruminant; lane 4, pork; lane 5, poultry; lane 6, soy bean; lane 7, maize; lane 8, control reagent.

The assay sensitivity was evaluated depending on PCR product intensity as it can be visualized in agarose gel. The DNA extracted sample consisted of a DNA mix of the four species was diluted in maize DNA for obtaining templates with known percent concentrations of fish material. Starting from the considered 100 % DNA template, seven serial DNA dilutions were performed: 10%, 1%, 0.1%, 0.01%, 0.005% 0.002% and 0.001% respectively; all of them were used as DNA template for PCR amplification (Fig. 2).

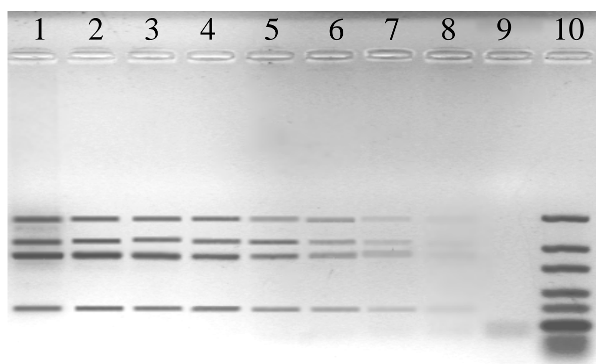

Fig. 2. Evaluation of assay sensitivity: progressive dilution of a mixed DNA template diluted in DNA of maize. Lane 1, 100%; lane 2, 10%; lane 3, 1%; lane 4, 0.1%; lane 5, 0.01%; lane 6, 0.005%; lane 7, 0.002%; lane 8, 0.001%; lane 9, molecular weight marker: O'GeneRuler Low Range DNA Ladder, Fermentas.

Evaluating the intensity of PCR products from *Figure 2*, it is nearly impossible to discriminate among concentrations animal DNA above 0.1%. However, as expected, the intensity decreases gradually at lower concentrations, tending to decrease dramatically in case of 0.001% concentration.

In the case of 0.001% diluted sample the amplicons are still perceptible meaning that this concentration can be considered a lower threshold for the sensitivity of this multiplex PCR assay.

To test the sensibility of the four animal species detection in commercial product matrices, the samples were submitted to specific simplex PCR assay by using the different four primers in four separate reactions (Fig. 3, 4, 5, 6).

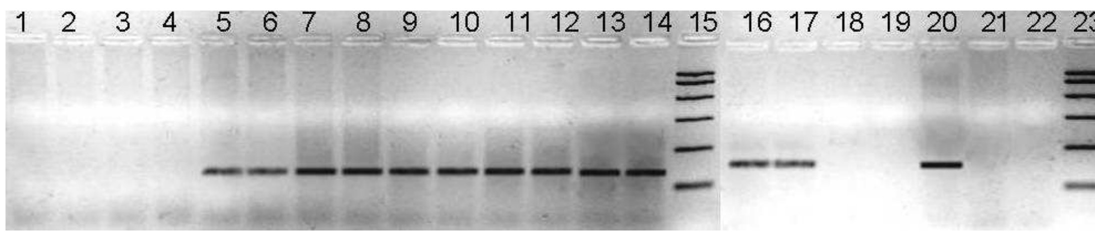

Fig. 3. Cattle species detection in commercial product by simplex PCR assay: Lane 1 – 2, A; lane 3 – 4, B; lane 5 – 6 C; lane 7 – 8, D ; lane 9 – 10, E ; lane 11 – 12, F; lane 13 – 14, G; lane 15, molecular weight marker: PCR marker, *Promega*; lane 16 – 17, H; lane 18, extraction control - EC; lane 19, environment blank control – EB; lane 20, positive control - ruminant DNA template; lane 21, negative template control (maize DNA); lane 22 control reagent; lane 23, molecular weight marker: PCR marker, *Promega.*

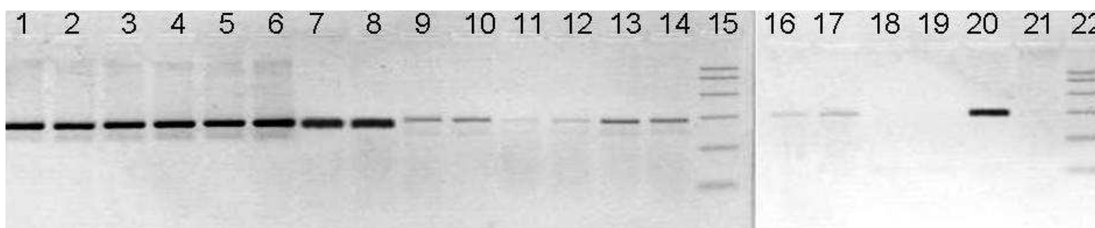

Fig. 4. Pork species detection in commercial product by simplex PCR assay: Lane 1 – 2, A ; lane 3 – 4, B ; lane 5 – 6 C; lane 7 – 8, D; lane 9 – 10, E ; lane 11 – 12, F; lane 13 – 14, G; lane 15, molecular weight marker: PCR marker, *Promega*; lane 16 – 17, H; lane 18, extraction control - EC; lane 19, environment blank control – EB; lane 20, positive control - ruminant DNA template; lane 21 control reagent; lane 22, molecular weight marker: PCR marker, *Promega.*

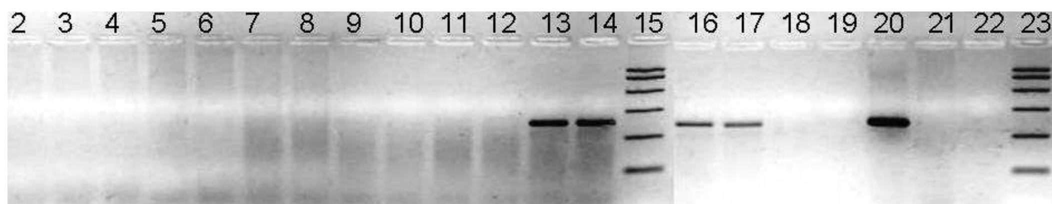

Fig. 5. Fish species detection in commercial product by simplex PCR assay: Lane 1 – 2, A ; lane 3 – 4, B; lane 5 – 6 C; lane 7 – 8, D ; lane 9 – 10, E; lane 11 – 12, F; lane 13 – 14, G; lane 15, molecular weight marker: PCR marker, *Promega*; lane 16 – 17, H; lane 18, extraction control - EC; lane 19, environment blank control – EB; lane 20, positive control - ruminant DNA template; lane 21, negative template control (maize DNA); lane 22 control reagent; lane 23, molecular weight marker: PCR marker, *Promega.*

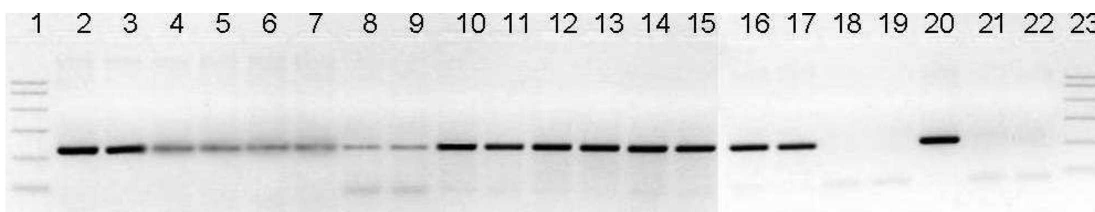

Fig. 6. Poultry species detection in commercial product by simplex PCR assay: Lane 1, molecular weight marker: PCR marker, *Promega* 2 – 3, A ; lane 4 – 5, B ; lane 6 – 7 C; lane 8 – 9, D ; lane 10 – 11, E ; lane 12 – 13, F; lane 14 – 15, G ; lane 16 – 17, H ; lane 18, extraction control - EC; lane 19, environment blank control – EB; lane 20, positive control - poultry DNA template; lane 21, negative template control (maize DNA); lane 21 control reagent; lane 22, molecular weight marker: PCR marker, *Promega.*

The results obtained following the four separate amplifications, compared to the labeled components are listed in *Table 3*.

Tab. 3

Results of the identification assay

Samples	Label	Result
Sausages (A)	Chicken	Chicken/pork
Processed meat product (B)	Pork	Pork/ poultry
Processed meat product (C)	Pork/others	Pork/beef/ poultry
Raw meat product (D)	Pork/beef	Beef/pork/poultry
Pet food (E)	Bovine/turkey	Beef/turkey/pork
Pet food (F)	Chicken/lamb	Chicken/lamb/pork
Pet food (G)	Chicken/others	Chicken/fish/pork
Pet food (H)	Fish/others	Fish/poultry/pork

Further on the samples were submitted to multiplex PCR, to determine its specificity when DNA extracted from commercial raw materials was analyzed (Fig. 7).

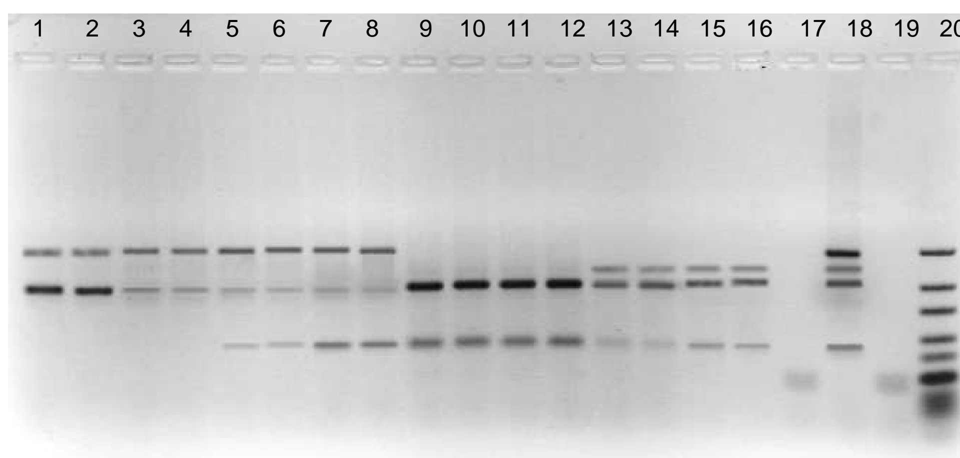

Fig. 7. PCR analysis animal species detection on commercial meat products and pet foods. Lane 1 – 2, A; lane 3 – 4, B ; lane 5 – 6 C; lane 7 – 8, D ; lane 9 – 10, E ; lane 11 – 12, F; lane 13 – 14, G ; lane 15 – 16, H; lane 17, control, negative DNA (maize) template; lane 18, control, positive DNA template (mix of ruminant, pork, poultry and fish); lane 19, control reagent; lane 20, molecular weight marker: O'GeneRuler Low

Analyzing the gel it turned out that all of the positive samples identified by simplex PCR reactions were positive in the multiplex reaction too. Therefore, the applicability of the assay to commercial meat products and pet food has been demonstrated.

The pointed out results report the true species composition of the listed sample products. With regard to commercial meat products, the label has been confirmed by the DNA analysis only for the C sample - processed meat product.

For pet food, the claimed species has always been detected, but also the presence of pork contamination was emphasized.

CONCLUSIONS

The multiplex PCR described in this paper proved to be very sensitive, with a very low detection limit when DNA mixtures were tested.

The method is also very sensitive and reliable in species identification. It describes a simple and promising method for identification of animal residuals in industrial meat products by a single PCR reaction. The test could be useful and applied by researchers and quality control laboratories for verification and control of industrial meat products and other food and feed stuff to verify the origin of the raw materials.

REFERENCES

1. Bottero, M.T., Dalmasso, A., Nucera, D., Turi, R.M., Rosati, S., Squadrone, S., Goria, M., Civera, T., (2003). Development of a PCR assay for the detection of animal tissues in ruminant feeds. Journal of Food Prot; 66. in press.
2. Dalmasso, A., Fontanella, E., Piatti, P., Civera, T., Rosati S., Bottero, M.T., (2004). A multiplex PCR assay for the identification of animal species in feedstuffs, Molecular and Cellular Probes 18, 81–87.
3. European Commission. Commission decision of 27 March 2002 amending council decision 2000/766/EC and commission decision 2001/9/EC with regard to transmissible spongiform encephalopathies and the feeding of animal proteins. (2002/248/EC). Official Journal of European Communities 2002; L84(28/3/2002):71–2.
4. Ghovvati, S., Nassiri, M.R., Mirhoseini, S.Z., Heravi Moussavi, A., Javadmanesh A. (2009). Fraud identification in industrial meat products by multiplex PCR assay, Food Control 20 696–699.
5. Gouli, Z., Mingguang, Z., Zhijiang, Z., Hongsheng, O. and L. (1999). Establishment of a polymerase chain reaction for the identification of beef. *Meat Sci*. 48: 233-236.
6. Nierderer, M. and Bolhalder, R. (2001). Identification of species-specific Central nervous system by gas chromatography-mass spectrometry (GC – MS) a possible method for supervision of meat products and cosmetics. *Mitteleilungen aus dem Gebeite der Lebensmitteluntersuchung and hygiene*. 92: 133-144.
7. Parodi, B., Aresu, O., Bini, D., Lorenzini, R., Schena, F., Visconti, P., Cesaro, M., Ferrera, D., Andreotti, V. and Ruzzon, T. (2002). Species identification and confirmation of human and animal cell lines: a PCR based approach. *Biotechniques.* 32: 433-440.
8. Querci, M., Jermini, M., Van den Eede, G., (2004).The Analysis Of Food Samples For The Presence Of Genetically Modified Organisms, User Manual, European Commission, Joint Research Centre, Special Publication No1.03.114.