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POLYMERASE CHAIN REACTION DETECTION OF RABBIT DNA IN FOOD AND ANIMAL FEED

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Abstract: A polymerase chain reaction (PCR) based on oligonucleotide primers targeting the mitochondrial 12S rRNA gene was developed for the specific identification of rabbit DNA (*Oryctolagus cuniculus*) in food and feedstuffs. The specificity of the primers was verified by PCR analysis of DNA from 32 non-target species including mammals, birds, fish, and plant species. Analysis of experimental mixtures demonstrated the presence of rabbit-derived materials in the range of 0.1-100%. Prolonged heat treatment (up to 133ºC for 20 min at 300 kPa) applied to rabbit muscle/oats binary mixtures did not affect the performance of the method, which could therefore be said to be very useful for the accurate identification of rabbit materials in products submitted to denaturing technologies when other methods are not suitable.

Key Words: rabbit, feedstuff, PCR, species identification, 12S rRNA gene.

INTRODUCTION

The European Union has adopted strong measures to avoid the spread of transmissible spongiform encephalopathies (TSE) in recent years. For this reason, feeding of processed animal protein (PAPs) to farm animals reared for production of food is forbidden (EC Council, 2001), and Regulation 2002/1774/EC (EC Council, 2002) imposes a ban on feeding animals with proteins from the same species. Moreover, to enforce control measures on the authentication of meat, the legislative authority establishes that meat products must be accurately labelled regarding species content (Pascal and Mahé, 2001). However it is not always easy to identify animal species in food. Animal meats often undergo a great deal of processing before being sold. The end product rarely resembles its whole animal predecessor, making it extremely difficult to distinguish between species that might be present, often allowing food authenticity to go unchecked (Schlumpberger, 2004). For these reasons, the development of accurate methods for rapid identification of animal materials in food and feedstuffs is essential to protect consumers and also to enforce feed bans.

DNA-based methods have become essential tools for species identification in animal products and feedstuffs, and are widely used nowadays (Teletchea *et al*., 2005). In particular, the polymerase chain reaction (PCR) methodology using specific PCR primers is a widely applied approach for rapid detection and identification of organisms at species and intraspecies level (Matsunaga *et al*., 1999; Herman, 2001; Rodríguez *et al*., 2003).

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The vast majority of PCR applications that use specific primers for meat species identification published to date are focused on domestic species such as cattle, sheep, goats, pigs, turkeys, chickens, ducks and geese (Colgan *et al*., 2001; Dalmasso *et al*., 2004; Di Pinto *et al*., 2005). A few procedures have been developed for the identification of cat, dog, rat or horse tissues (Chisholm *et al*., 2005; Martín *et al*., 2007b) employing species-specific primers. However, even fewer studies have been reported to date on rabbit meat authentication employing polymerase chain reaction (PCR) coupled with complementary techniques. The PCR-RFLP approach amplifying a cytochrome b gene region developed by Partis *et al*. (2000) detected rabbit DNA extracted from cooked and uncooked tissues. Arslan *et al*. (2005) developed a random amplified polymorphic DNA (RAPD) method using a 10-base primer for the identification of raw meats from different species including rabbits. Walker *et al*. (2004) designed a PCR-based assay for the identification and quantification of equine, canine, feline, rat, hamster, guinea pig, and rabbit meat using amplification of genome-specific short and long interspersed elements.

In this work, specific primers were developed based on the 12S ribosomal RNA mitochondrial gene to attempt PCR detection and identification of rabbit DNA in food and feedstuffs.

MATERIALS AND METHODS

Sample Selection

The animal and plant species analysed in this work are shown in Table 1. All animal samples were provided by a local slaughterhouse or by The Veterinary Hospital (Facultad de Veterinaria, Universidad Complutense de Madrid, Spain). Samples were morphologically identified by trained veterinarians and transported to the laboratory under refrigeration.

In order to evaluate the test sensitivity, five different percentages, 0.1, 1, 5, 10 and 25% (w/w) of rabbit muscle tissues in oats were prepared with a final weight of 100 g. Two hundred mL of sterile phosphate-buffered saline (PBS; 136 mM NaCl, 1.4 mM KH_2PO_4, 8.09 mM $Na_2HPO_4.12H_2O$, and 2.6 mM KCl, pH 7.2) were added to the binary mixtures, which were homogenized by a blender (Sunbeam Oster, Florida, USA). A pure rabbit sample was used as positive control.

The effect of thermal treatments on the technique's ability to identify the target species was checked through the analysis of binary mixtures prepared with heat-treated tissues. To prepare the heat-treated mixtures, 100 g of rabbit muscle was finely chopped and processed in an autoclave in compliance with European legislation (EC Council, 2002). Three different heat treatments were applied: 120ºC for 50 min, 110ºC for 120 min, and 133ºC at 300 kPa for 20 min. Temperature was checked by introducing a temperature data logger, mod EDI-85A/125A (Ebro Electronic GMBH & Co, Ingolstadt, Germany) in the autoclave, together with the rabbit muscle. The autoclaved tissues were let cool at room temperature, and were used to prepare the rabbit muscle/oats mixtures containing from 0.1 to 25% rabbit component. Raw and heat-treated binary mixtures were processed directly or stored at −20ºC until used.

PCR amplification and sequencing of a conserved fragment in the mitochondrial 12S rRNA region and design of rabbit-specific primers

Amplification and sequencing of a conserved 12S rRNA gene fragment (~720 bp) from rabbit and Cape hare meats was accomplished following a previously described procedure (Martín *et al*., 2007a). The set of primers used for this purpose were: 12S-FW and 12S-REV oligonucleotides (Fajardo *et al*., 2006).

Information obtained after alignment of 12S rRNA gene sequences from various animal and plant species available in the Genbank database, together with the 720 bp amplicons obtained from rabbit (FM164771, FM164772) and Cape hare (FM164770) with the 12S-FW and 12S-REV set of primers was used to

Table 1: Specificity of the 12SpRabbDIR/12SpRabbINV primers designed for the specific detection of rabbit tissues using DNA obtained from several animal and plant species. 18SpEUDIR/18SpEUINV are positive control primers.

Common name	Scientific name	Rabbit-specific PCR system	Positive control PCR
Rabbit	*Oryctolagus cuniculus*	110 bp	140 bp
Cape hare	*Lepus capensis*	-[1]	140 bp
Chicken	*Gallus gallus*	-	140 bp
Turkey	*Meleagris gallipavo*	-	140 bp
Duck	*Anas platyrhynchos x Cairina muschata*	-	140 bp
Goose	*Anser anser*	-	140 bp
Cattle	*Bos taurus*	-	140 bp
Sheep	*Ovis aries*	-	140 bp
Goat	*Capra hircus*	-	140 bp
Pig	*Sus scrofa domestica*	-	140 bp
Horse	*Equus caballus*	-	140 bp
Cat	*Felis catus*	-	140 bp
Dog	*Canis familiaris*	-	140 bp
Rat	*Rattus norvegicus*	-	140 bp
Mouse	*Mus musculus*	-	140 bp
Anchovy	*Engraulis encrasicolus*	-	140 bp
Atlantic salmon	*Salmo salar*	-	140 bp
Hake	*Merluccius spp*	-	140 bp
Grouper	*Epinephelus marginatus*	-	140 bp
Nile perch	*Lates niloticus*	-	140 bp
Rainbow trout	*Oncorhynchus mykiss*	-	140 bp
Sardine	*Sardina pilchardus*	-	140 bp
Sea bass	*Dicentrarchus labrax*	-	140 bp
Tuna	*Thunnus spp*	-	140 bp
Wreck fish	*Polyprion americanus*	-	140 bp
Barley	*Hordeum vulgare*	-	140 bp
Rice	*Oryza sativa*	-	140 bp
Maize	*Zea mays*	-	140 bp
Oats	*Avena sativa*	-	140 bp
Sunflower	*Helianthus annuus*	-	140 bp
Soybean	*Glycine max*	-	140 bp
Rye	*Secale cereale*	-	140 bp
Wheat	*Triticum aestiuum*	-	140 bp

[1]Means no amplification of the PCR product.

design the rabbit-specific primers 12SpRabbDIR (5´-CAAAAGTAAGCTCAATTACCACCGTA-3´) and 12SpRabbINV (5´-ATAAGGGCTTTCGTATATTCGGAA-3´) to amplify a 110 bp fragment of the 12S rRNA gene in rabbit (*Oryctolagus cuniculus*) DNA. The primer pair 18SpEUDIR/18SpEUINV (Martín *et al*., 2008) was used for amplification of a conserved region of 140 bp of the 18S rRNA gene in all the animal and plant species commonly used in feedstuffs. The EMBOSS software package version 2.2.0 and Primer Express 2.0 software (Perkin-Elmer/Applied Biosystems Division, Foster city, CA,USA) were used for alignments and primer design.

PCR Amplification

Genomic DNA was obtained from 200 mg of animal, plant or binary mixture materials using a Wizard DNA Clean-up System kit (Promega Corp., Madison, WI) as described by Martín *et al*. (2007a).

Integrity of the DNA obtained from raw and heated muscle was checked by agarose gel electrophoresis in a 1.5% low electroendosmosis D1 agarose gel (Hispanlab S. A., Torrejon, Spain) containing 1 µg/mL ethidium bromide in Tris-acetate buffer (0.04 M Tris-acetate and 0.001 M EDTA, pH 8.0). Electrophoresis was performed at 85 V for 1 h.

Amplification reactions were carried out on a total volume of 25 µL containing 125 ng of template DNA, 2 mM $MgCl_2$, 12.5 pmol of each primer, 200 µM of each dNTP and 1U of Tth DNA polymerase (Biotools, Madrid, Spain) in a reaction buffer supplied with the enzyme. Amplification was performed in a Progene thermal cycler (Techne Ltd., Cambridge, UK) using the following conditions: an initial heat denaturation step at 93ºC for 2 min, followed by 35 cycles consisting of 30 s at 93ºC for DNA denaturation, 30 s for primers annealing, and 45 s at 72ºC for DNA extension. The last extension step at 72ºC was maintained for 3 min. Annealing temperature was optimized to 63ºC for rabbit and 65ºC for eukaryote primers.

The 12S rRNA and 18S rRNA amplicons (10 µL) were mixed with 2 µL of Gel Loading Solution (Sigma), and analyzed by electrophoresis in a 3.5% MS-8 high resolution agarose gel (Hispanlab S. A., Torrejón, Spain).

RESULTS AND DISCUSSION

Molecular techniques developed over the last two decades have allowed the identification of animal species in raw or processed meat products. Among DNA-based techniques, PCR using species-specific primers offers the advantages of being cheaper, faster, and more appropriate for species identification in meat products submitted to different processing treatments and in the analysis of admixed meats including two or more species in their composition (Fajardo *et al*., 2007).

In this study, the mitochondrial 12S rRNA gene was selected as marker to design specific primers for the amplification of rabbit material since it has an acceptable length, an adequate level of mutation and there is a wide spectrum of sequences available in the databases (Rodríguez *et al*., 2004).

Accurate species identification by PCR is greatly dependent on the specificity of the primers used, which should hybridize to a DNA segment with sufficient species-to-species variation (Kusama *et al*., 2004). To fulfill the PCR identification requirements, sequence data from 12S rRNA amplicons obtained from a hare and from two rabbits were sequenced and aligned together with sequences from several animal species available in the GenBank database. The BLAST analysis of the sequences produced a percentage of identity of 99% and 97% between the individuals analysed (accession numbers FM164771 and FM164772) and the *Oryctolagus cuniculus* complete mitochondrial genome (accession number AJ001588) available in the database. The similarity of the rabbit sequences with those of hare (Lepus spp.) was 91% and 92%, respectively.

Because a large number animal species can be included in compound feedstuffs, to ensure the specificity of the rabbit primers designed (12SpRabbDIR and 12SpRabbINV) and exclude non-desired amplifications, tests were carried out on DNA obtained from 30 rabbit muscular samples, 24 non-target animal species and 8 plant species. Specific DNA fragments of 110 bp DNA were successfully amplified for all 30 rabbit samples, and no cross-species amplification was observed for the other animal and plant species analyzed (Table 1).

In order to avoid false-negative results due to a failure of the amplification procedure we used a positive control primer pair, 18SpEUDIR and 18SpEUINV, that amplified a conserved region of 140 bp of the 18S rRNA gene in all the plant and animal species tested (Table 1).

It is known that a critical factor in PCR analysis is the heat denaturation of DNA subsequent to the processing treatments applied to certain products. The standard rendering condition used to produce meat and bone meal (EC Council, 2002) causes denaturation of prions, but also affects DNA stability. As shown in Figure 1, total DNA extracted from raw tissues (lane 1) exhibited a molecular weight of about 12 kbp, whereas DNA extracted from heat-treated tissues exhibited very low molecular weight compared with raw tissues: less than 600 bp for tissues heated at 110ºC/120 min (lane 2), and less than 200 bp for tissues heated at 120ºC/50 min (lane 3) or 133ºC/300 kPa/20 min (lane 4). DNA degradation has been shown to cause failures in PCR protocols, mainly when the fragments to be amplified are large (Matsunaga *et al*., 1999; Hird *et al*., 2006). In this context, PCR assays of food matrices in which thermal effects may degrade the DNA should rely preferably on primers targeting short DNA fragments (less than 200 bp).

The sensitivity of the rabbit-specific assay (lowest amount of rabbit tissues in a feed or food producing visible DNA amplification) was evaluated by PCR amplification of DNA obtained from binary mixtures containing 0.1, 1, 5, 10, 25 and 100% (w/w) of rabbit muscle in an oats matrix. It was observed that the lower the percentage of rabbit muscle in the admixture, the fainter the band obtained in the PCR with the species-specific primers. The lowest percentage producing visible DNA amplification using the

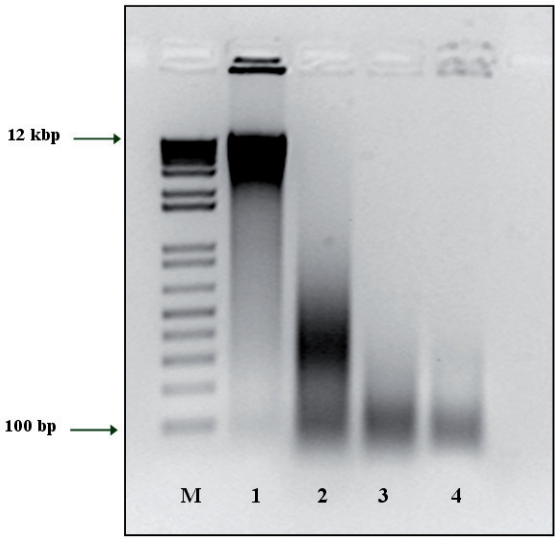

Figure 1: Gel electrophoresis of total genomic DNA extracted from rabbit muscle. Sample in lane 1 was obtained from raw muscle, and samples in lanes 2, 3 and 4 were obtained from rabbit muscle heat treated at: 110ºC/120 min (2), 120ºC/50 min (3), and at 133ºC/300 kPa/20 min (4). 5 µL of undiluted DNA were loaded in each lane. M, molecular weight marker 1kb plus DNA ladder (GibcoBRL).

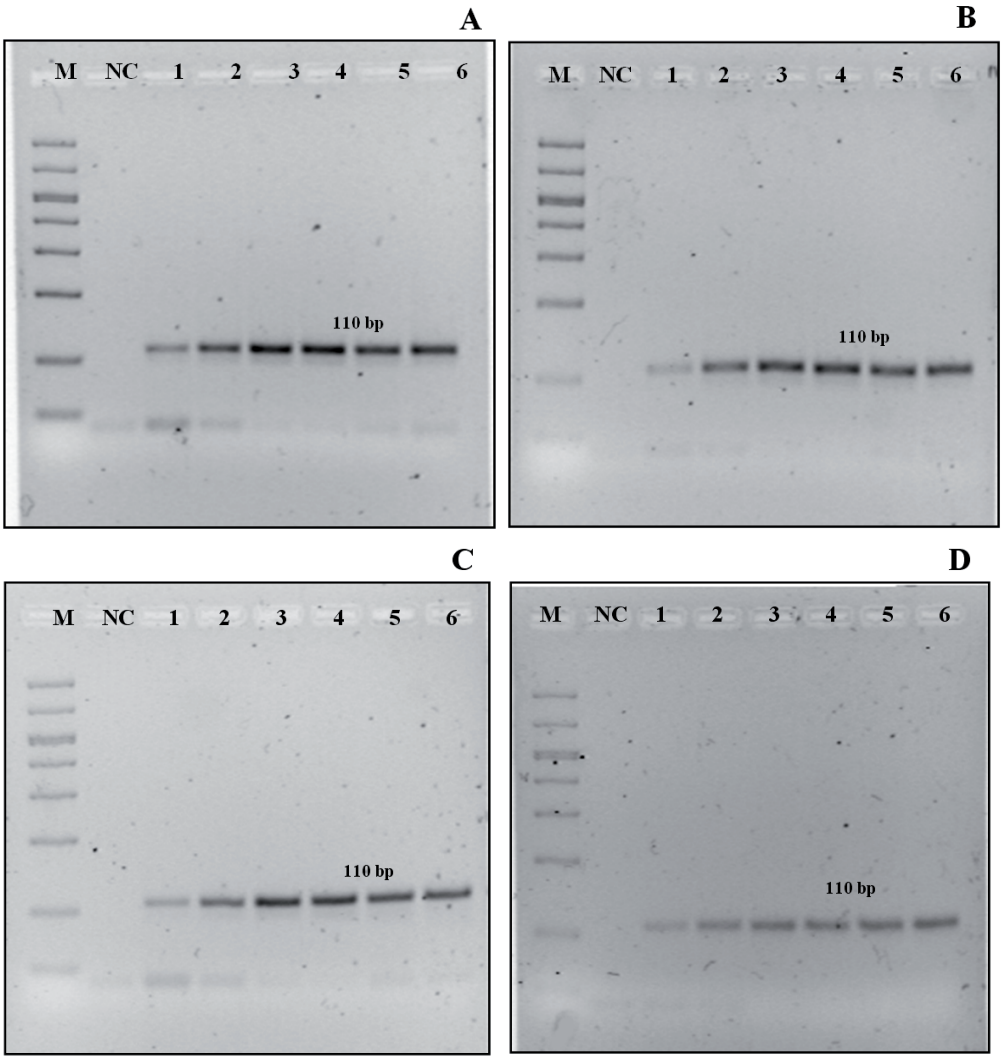

Figure 2: Electrophoretic analysis of the 12S rRNA PCR products obtained from raw and heat treated binary rabbit muscle/oats mixtures, using primers 12SpRabbDIR and 12SpRabbINV. Lanes 1 to 6 are samples of binary mixtures containing 0.1, 1, 5, 10, 25 and 100% of rabbit muscle, respectively. (A) raw rabbit muscle in oats; (B) rabbit muscle heated at 110ºC/120 min in oats; (C) rabbit muscle heated at 120ºC/50 min in oats; (D) rabbit muscle heated at 133ºC/300 kPa/20 min in oats. M, Molecular weight marker 50-1000 bp ladder (Biomarker® Low, BioVentures, Inc.); NC, negative control. The pictures are reverse images of the 3.5% MS8 agarose gels containing ethidium bromide.

primer pair 12SpRabbDIR and 12SpRabbINV was 0.1% for raw muscle/oats mixtures (Figure 2A). The steam treatments applied to binary mixtures did not modify the detection limit. Thus, similar results were obtained for binary mixtures submitted to 110ºC/120 min (Figure 2B), 120ºC/50 min (Figure 2C), and 133ºC/300 kPa/20 min (Figure 2D).

There are only a few reports on the successful identification of rabbit meats (Hunt *et al*., 1997; Partis *et al*., 2000; Walker *et al*., 2004; Arslan *et al*., 2005). The nonradioactive slot blot developed by Hunt *et al*.

(1997) identifies rabbit in both raw and commercially canned products, but the detection limit of the assay was 2.5%. The PCR-RFLP approach developed by Partis *et al*. (2000) detected rabbit DNA extracted from cooked and uncooked tissues, but was not appropriate for the analysis of complex mixtures. Walker *et al*. (2004) designed a PCR-based assay for the quantification of rabbit meat using amplification of genome-specific short and long interspersed elements. The minimum effective quantitation level of this assay was 1% when testing DNA samples from mixed sources. The random amplified polymorphic DNA method developed by Arslan *et al*. (2005) could not detect heat-treated rabbit meats.

Compared to previous PCR methods, the advantages of the proposed PCR assay include being faster and more appropriate for rabbit tissue identification in complex mixtures (patés, minced meat products, etc.) with two or more species in their composition. It has also been demonstrated to be useful for the analysis of products subjected to the severe heat treatment used to produce meat and bone meal (133ºC/300 kPa/20 min).

CONCLUSIONS

It can be concluded that the PCR assay described in this paper is an appropriate method of testing for the presence of low levels (0.1%) of rabbit tissues, and could be used in inspection programs to detect undeclared ingredients in foods and animal feeds, even in samples that have been subjected to severe heat/pressure treatment, when other methods are not suitable.

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