Direct and Highly Species-Specific Detection of Pork Meat and Fat in Meat Products by PCR Amplification of Mitochondrial DNA

J. F. Montiel-Sosa,[†,‡] E. Ruiz-Pesini,[†] J. Montoya,[†] P. Roncalés,[§] M. J. López-Pérez,*[,†] and A. Pérez-Martos[†]

Departamento de Bioquímica y Biología Molecular y Celular, Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, E-50013 Zaragoza, Spain, and Departamento de Ciencias Biológicas, Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autonoma de México, Cuautitlán Izcalli Estado de México, Mexico

Highly species-specific primers for pork D-loop mtDNA have been designed. Use of these and restrictive PCR amplification conditions has improved a reliable and rapid method for detecting a PCR-amplified 531 bp band from pork. It has been proved useful for detecting both pork meat and fat in meat mixtures, including those dry-cured and heated by cooking. Absence of response in PCR-amplified samples or mixtures from bovine, ovine, chicken, and human was also demonstrated. Furthermore, wild boar and pork samples can be also easily distinguished by a simple *Ava*II restriction analysis.

Keywords: *Mitochondrial DNA; PCR analysis; pork; meat; meat products; species identification*

INTRODUCTION

Interspecies meat adulteration or preparation of meat products by mixing meats and fats of different origin is a common procedure in most countries. These facts are of major concern for many consumers, particularly in relation with ambiguous or improper labeling, adulteration with cheaper meats, or religious specifications such as "kosher" food for Jews and Muslim prohibitions. Species identification is known to be especially difficult when meat products have been thermally processed, due to protein denaturation. A significant problem in its own, too, is the use of pork fat in beef or fowl meat products. Therefore, there is a need for rapid and reliable methods for species identification in such varieties of food commodities.

Methods currently available for species identification include protein isoelectric focusing in polyacrylamide gels (King and Kurth, 1982) and agar gel immunodiffusion assays (Berger et al., 1988). Serological tests are specific and sensitive, but cross-reactions of closely related species cannot be ruled out. These problems can be solved with methods based on the presence of species-specific DNA sequences in meat detected by DNA hybridization (Wintero et al., 1990; Chikuni et al., 1990) or Polymerase Chain Reaction (PCR) (Saiki et al., 1988).

The use of mtDNA for PCR identification of species may offer a series of advantages. First, mtDNA genes are present in thousands of copies per cell. This fact should improve the possibility of amplifying template molecules of adequate size among the DNA fragments brought about by heat denaturation. Second, vast knowledge on animal mtDNA gene organization, as well as the availability of reported sequences in many species, makes also possible an easy design of specific primers for amplification. Third, the large variability of mtDNA allows reliable identification of precise species in mixtures. Finally, intraspecific variability of mtDNA offers the possibility of discriminating breeds currently used in industrial swine production. Thus, sequencing or restriction fragment length polymorphism (RFLP) of mtDNA PCR fragments has been already used to identify different species of canned tuna (Quintero et al., 1998), bonito (Ram et al., 1996), or snails (Borgo et al., 1996). The use of specifically designed primers under restrictive conditions of PCR amplification could also make possible the direct and specific identification of PCR-amplified mtDNA fragments, avoiding subsequent sequencing or RFLP identification (Matsunaga et al., 1999).

The main target of this work consisted of improving a reliable method to detect pork in heated meat products by a simple band identification of PCR products. Detection of pork by PCR amplification of nuclear 18S ribosomal RNA and growth hormone genes or Y chromosome has been previously described (Meyer et al., 1994, 1995; Meer and Eddinger, 1996). The sequence of porcine mtDNA has been previously reported (Ghivizzani et al., 1993; Ursing and Arnason, 1998), so we have designed species-specific primers and restrictive PCR amplification conditions for pork D-loop mtDNA that allow the identification of pork meat and fat, even heated, by the presence of an amplified band of 531 bp. This band is absent in PCR-amplified products from bovine, ovine, chicken, and human samples. Wild boar and pork amplified DNA can be easily distinguished by digestion with *Ava*II restriction enzyme.

MATERIALS AND METHODS

Meat and Meat Product Samples. Samples of fresh meat (pork, wild boar, chicken, lamb, and beef), fat (pork fat), and

* Address correspondence to this author at the Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Veterinaria, Miguel Servet 177, 50013 Zaragoza, Spain (fax 34-976761612; e-mail lopezper@posta.unizar.es).

[†] Departamento de Bioquímica y Biología Molecular y Celular.

[‡] Departamento de Ciencias Biológicas.

[§] Departamento de Producción Animal y Ciencia de los Alimentos.

10.1021/jf9907438 CCC: $19.00
Published on Web 06/09/2000

meat products (mortadella, pork sausage, and dry-cured ham) were purchased in a local retail market. Pork/beef mixtures were prepared by thoroughly mixing various percentages (5, 10, 20, 50, 75, and 100) of pork and beef previously ground on a meat grinder (Moulinex, Urnieta, Spain).

Extraction of Total DNA. The samples (0.5 g) were crushed in liquid nitrogen, and the powder was homogenized with 0.5 mL of RSB buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 25 mM EDTA) and incubated for 3 h at 50 °C with 20 μL of 25% SDS and 1 μL proteinase K (200 μg/mL). Human DNA was prepared according to the same procedure from pelleted cells coming from 5 mL of blood diluted with 45 mL of water. Total DNA was isolated by two extractions with phenol/chloroform/isoamyl alcohol (25:25:1) and ethanol precipitation.

Mitochondrial DNA Amplification. mtDNA D-loop region fragments were amplified by PCR, with the total DNA that contained mtDNA as templates. The PCR primers were designed after comparison of the published nucleotide sequences of the D-loop region of porcine, ovine, bovine, and chicken, and then porcine-specific sequences were chosen. The primer sequences used were as follows:

pig F 5′ AACCCTATGTACGTCGTGCAT (15592)

pig R 5′ ACCATTGACTGAATAGCACCT (16124)

Amplifications of DNA were carried out in a final volume of 50 μL in tubes containing 0.5 μg total of DNA, 10× reaction buffer (Sigma, St. Louis, MO), 10 mM each dATP, dCTP, dGTP, and dTTP (Boehringer Mannhein, Germany), 2.5 mM each primer (Gibco BRL Life Technologies, Paisley, Scotland), and 2 units of Taq DNA polymerase (Sigma). After 3 min of initial denaturation at 92 °C, 30 cycles of amplification at 92 °C for 20 s (denaturation), 58 °C for 20 s (hybridization), and 72 °C for 30 s (elongation) were carried out with a thermal cycler (Biometra T3, Göttingen, Germany). Amplification products were electrophoresed through 2% agarose gels in Tris-acetate buffer, pH 8.0, and made visible by staining with ethidium bromide and UV transillumination.

To distinguish between pork and wild boar, the amplified mtDNA was digested with 1 μL of *Ava*II restriction enzyme (5 units/μL) (Gibco BRL Life Technologies) for 4 h at 37 °C.

RESULTS

Takeda et al. (1995) had already described amplification of pork mtDNA by using primers that resulted in a band of 227 bp. We had previously found that a band of similar size was also obtained from bovine mtDNA by using the same primers (data not shown). An analysis of the bovine mtDNA sequence (Anderson et al., 1982) showed that those primers could hybridize with bovine mt DNA at positions 16060 and 16320 (15−16 of 21 nucleotides coincide) that could result in a 260 bp band. To avoid such heterologous hybridization, we designed new primers, designated pig F and pig R, which would result in an amplification fragment of 531 bp. These primers should not produce any amplified fragment by sequence coincidence higher than 14 of 21 nucleotides in the sequence of bovine (Anderson et al., 1982), ovine (Zardoya et al., 1995), or chicken (Desjardins and Morais, 1990) mtDNA. Figure 1 shows that a 531 bp band was obtained from pork (lane e) and wild boar (lane f) but not from bovine, chicken, ovine, and human mtDNA (lanes a−d).

An increase of denaturing and hybridizing temperatures to 95 and 60 °C, respectively, produced an amplified DNA band of the same size in pork and wild boar but not in the other species. However, we decided to use

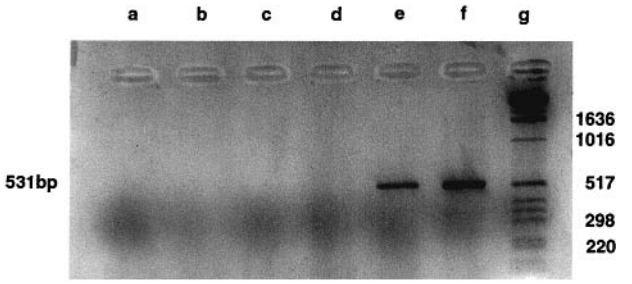

Figure 1. Electrophoresis analysis in 2% agarose gels of the PCR products obtained with beef (a), chicken (b), lamb (c), human (d), wild boar (e), and pork (f) DNA. Lane g is a 1 kb ladder DNA marker.

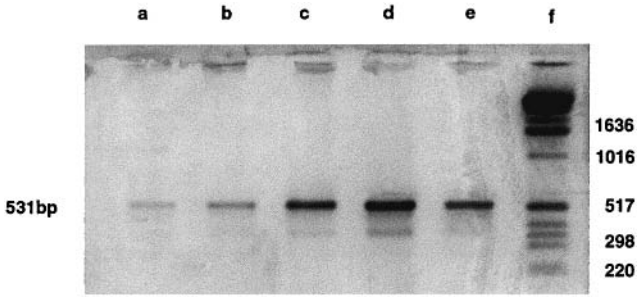

Figure 2. Electrophoresis analysis in 2% agarose gels of the PCR amplification obtained with pork derivative products: (lanes a−d) mortadella, fat pork, pork sausage, and dry-cured ham, respectively; (lane e) pork; (lane f) 1 kb ladder DNA marker.

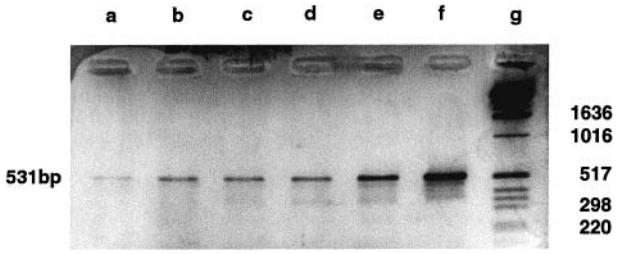

Figure 3. Electrophoresis analysis in 2% agarose gels of the PCR products obtained with mtDNA from different mixtures of pork and beef: (lanes a−f) 5% pork/95% beef, 10% pork/90% beef, 20% pork/80% beef, 50% pork/50% beef, 75% pork/25% beef, and 100% pork, respectively; (lane g) 1 kb ladder marker.

the more restrictive conditions to ensure a more specific amplification. The same amplified 531 bp band was observed in DNA obtained from mortadella, pork fat, pork sausage, and dry-cured ham (Figure 2).

To find out if the intensity of the band obtained by using pig F and pig R primers was reliable in a semiquantitative manner, DNA was prepared and amplified from different mixtures of bovine/porcine tissues (Figure 3), showing a visible correspondence between band intensity and porcine DNA amount used in each amplification. These results proved the absence of competitive events between bovine and porcine mtDNA that could avoid the amplification of pork DNA in mixtures of both species at least with >5% pork proportion.

To distinguish pork and wild boar samples, we sequenced the band amplified from both species with the primers used before (data not shown). The results showed that in wild boar there is deletion of one base at position 15879 with respect to pork mtDNA. This difference abolishes an *Ava*II restriction site. Therefore,

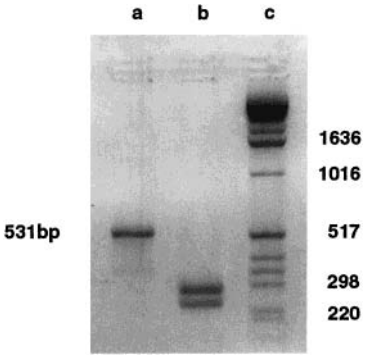

Figure 4. Electrophoresis analysis in 2% agarose gels of the products obtained with *Ava*II digestion of the amplified wild boar (lane a) and pork (lane b) mtDNA. Lane c is a 1 kb ladder marker.

the digestion of the 531 bp amplified DNA fragment produced the appearance of two bands of 286 and 245 bp in pork but not in wild boar (Figure 4).

DISCUSSION

The results presented in this work show that by using the pairs of primers for amplification of mtDNA designated pig F and pig R, together with the PCR conditions here described, a direct identification of pork meat and fat can be achieved. This identification appears to be highly species-specific, although the phylogenetically close subspecies of wild boar also gives rise to an amplified fragment of a very similar size. The sequences of the two primers designed were located in the D-loop region of porcine mtDNA, out of the well conserved regions CSB 1−3, and of the tandem repeat sequences present in porcine and intercalated between CSB 1 and 2. The amplified fragment of 531 bp is located in the highly variable region of the D-loop, close to the origin of replication of the heavy strand. The sequence analysis and the results obtained showed that these primers are highly specific for pork mtDNA. By using this method, porcine mtDNA was detected not only in fresh, spicy, salty (ham), or fat samples but also in minced and heated meat (mortadella). Furthermore, wild boar and pork samples can be easily distinguished by a simple restriction analysis.

These results confirm the appropriateness of mtDNA to species detection in the preparation of meat products, probably due, as stated above, to the high number of copies present per cell, which increases the probability of finding undegraded template molecules during amplification. The method described here allows an easy detection of the presence of pork meat and fat in meat products by a direct and simple identification of an electrophoretic band without requiring further RFLP or sequencing characterization. Furthermore, the presence of DNA from other species, such as bovine, did not competitively avoid the amplification of porcine DNA. Thus, this method can be used to detect fraud and to ensure that foods comply with religious regulations. In fact, a sensitivity of 5% allows a convenient detection of most adulterations with pork meat or fat based on cheapening practices.

Takeda et al. (1995) proved the existence of sequence polymorphism in the D-loop region of the mtDNA linked to different pig breeds. Some of these polymorphisms are included in the 531 bp fragment amplified with the set of primers here described and have been detected in our sequence analysis (data not shown). Therefore, further investigations should be carred out to characterize and associate these polymorphisms with the different pig breeds usually exploited for food production. Thus, RFLP of the 531 bp fragment here described would allow the identification of pig breed, even in heated meats. This is a matter of particular interest in the control and protection of foodstuffs possessing any quality label of geographical or specific origin.

We thank Santiago Morales for excellent technical assistance.

ABBREVIATIONS USED

PCR, Polymerase Chain Reaction; RFLP, restriction fragment length polymorphism.

LITERATURE CITED

Anderson, S.; De Bruij, A.; Coulson, I.; Eperon, C.; Sanger, F.; Young, I. Complete sequence of bovine mitochondrial DNA. *J. Mol. Biol.* **1982**, *156*, 683−717.

Berger, R; Mageau, K; Schwab, B.; Johnston, R. Detection of poultry and pork in cooked and canned meat foods by enzyme-linked immunosorbent assays. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 406−409.

Borgo, R.; Souty-Grosset, C.; Boucho, D.; Gomot, L. PCR-RFLP Analysis of mitochondrial DNA for identification of snail meat species. *J. Food Sci.* **1996**, *61*, 1−4.

Chikuni, K.; Ozutsumi, K.; Koishikawa, T.; Kato, S. Species identification of cooked meats by DNA hybridization assay. *Meat Sci.* **1990**, *27*, 119−128.

Desjardins, P.; Morais, R. Sequence and gene organization of the chicken mitochondrial genome. *J. Mol. Biol.* **1990**, *212*, 599−634.

Ghivizani, S. C.; Mackay, S. L. D.; Madsen, C. S.; Laipis, P. J.; Hauswirth, W. W. Transcribed heteroplasmic repeated sequences in the porcine mitochondrial DNA D-loop region. *J. Mol. Evol.* **1993**, *37*, 36−47.

King, N. L.; Kurth, L. Analysis of raw beef samples for adulterant meat species by enzyme staining of isoelectric focusing gels. *J. Food Sci.* **1982**, *47* (5), 1608−1612.

Matsunaga, T.; Chikuni, K.; Tanabe, R; Muroya, S.; Shibata, K.; Yamada, J.; Shinmura, Y. A quick and simple method for the identification of meat species and meat products by PCR assay. *Meat Sci.* **1999**, *51*, 143−148.

Meer, D. P.; Eddinger, T. J. Polymerase chain reaction for detection of male tissue in pork products. *Meat Sci.* **1996**, *44* (4), 285−291.

Meyer, R; Candrian, U.; Liithy, J. Detection of pork in heated meat products by the polymerase chain reaction. *J. Assoc. Off. Anal. Chem.* **1994**, *77*, 617−622.

Meyer, R; Höfelein, C.; Lüthy, J.; Candrian, U. Polymerase chain reaction-restriction fragment length polymorphism analysis: A simple method for species identification in food. *J. Assoc. Off. Anal. Chem.* **1995**, *78*, 1542−1551.

Quintero, J.; Sotelo, C. G.; Rehbein, H.; Pryde, S. E.; Medina, I.; Pérez-Martin, R. I.; Rey-Méndez, M.; Mackie, I. M. Use of mtDNA direct polymerase chain reaction (PCR) sequencing and PCR-restriction fragment length polymorphism methodologies in species identification of canned tuna. *J. Agric. Food Chem.* **1998**, *46*, 1662−1669.

Ram, J. L.; Ram, M. L.; Baidoun, F. F. Authentication of canned tuna and bonito by sequence and restriction site analysis of polymerase chain reaction products of mitochondrial DNA. *J. Agric. Food Chem.* **1996**, *44*, 2460−2467.

Saiki, R. K.; Gelfand, D. H.; Stoffel, S.; Scharf, S. J.; Hiyuchi, R; Horn, G. T.; Mullis, K. B.; Erlich, H. A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **1988**, *239*, 487−491.

Takeda, K.; Onishi, A.; Ishida, N.; Kawakami, K.; Komatsu, M.; Inumaru, S. SSCP analysis of pig mitochondrial DNA D-loop region polymorphism. *Anim. Genet.* **1995**, *26*, 321−326.

Ursing, B. M.; Arnason, U. The complete mitochondrial DNA sequence of the pig (*Sus scrofa*). *J. Mol. Evol.* **1998**, *47*, 302−306.

Wintero, A. K.; Thomsen, P. D. A comparison of DNA-hybridization, immunodiffusion, countercurrent immuno-electrophoresis and isoelectric focusing for detecting the admixture of pork to beef. *Meat Sci.* **1990**, *27*, 75−85.

Zardoya, R; Villalta, M.; López-Pérez, M. J.; Garrido-Pertierra, A.; Montoya, J.; Bautista, I. M. Nucleotide sequence of the sheep mitochondrial DNA D-loop and its flanking tRNA genes. *Curr. Genet.* **1995**, *28*, 94−96.

Received for review July 7, 1999. Revised manuscript received April 4, 2000. Accepted April 25, 2000. This work was supported by the Spanish Fondo de Investigaciones Sanitarias 98-1033, the Comisión Interministerial de Ciencia y Tecnologia (CICYT; ALI 96-587) and The National Council for Science and Technology (CONACYT) of Mexico.

JF9907438