Technical Note: Detection of cat, dog, and rat or mouse tissues in food and animal feed using species-specific polymerase chain reaction

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J ANIM SCI 2007, 85:2734-2739.
doi: 10.2527/jas.2007-0048 originally published online June 12, 2007

Technical Note: Detection of cat, dog, and rat or mouse tissues in food and animal feed using species-specific polymerase chain reaction[1]

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ABSTRACT: A PCR method based on the nucleotide sequence variation in the 12S ribosomal RNA, mitochondrial gene has been developed for the specific and qualitative detection and identification of cat, dog, and rat or mouse tissue in food and feedstuffs. The primers designed generated specific fragments of 108, 101, and 96 bp in length for cat, dog, and rat or mouse tissues, respectively. Specificity of the primers was tested against 32 nontarget species including mammals, birds, fish, and plant species. This PCR method allowed detection of raw and heated cat, dog, and rat or mouse tissues in meat/oats mixtures even when the concentration of the target species was reduced to 0.1%. Furthermore, the performance of the method was not affected by prolonged heat-treatment (up to 133°C for 20 min at 300 kPa), and consequently, it could be very useful to verify the origin of raw materials in food and feedstuffs submitted to denaturing technologies, for which other methods cannot be applied.

Key words: cat, dog, feedstuff, polymerase chain reaction, rat, mouse, species identification, 12S ribosomal ribonucleic acid gene

J. Anim. Sci. 2007. 85:2734–2739
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INTRODUCTION

To avoid spread of transmissible spongiform encephalopathies, feeding of processed animal protein to farm animals reared for production of food is forbidden in the European Union (European Commission, 2001). There are also regulations about animal by-products that may be used to produce pet food and dog chews (European Commission, 2002). Therefore, detection of animal tissues in feedstuffs is required to verify compliance with labeling requirements and feed traceability.

A microscopic method (European Commission, 1998), validated in 1998, became the official method used for determination of prohibited materials in animal feed. However, microscopy does not allow determination of the species origin of the material, and it is time-consuming (Armour and Blais, 2006). On the contrary, immunochemical and genetic techniques are capable of differentiating animal species, although protein-based techniques may be less sensitive in heat-treated material because of alteration of some specific epitopes (Hofmann, 1996). Disadvantages of protein-based methods can be solved with the use of genetic techniques such as PCR (Meyer et al., 1994; Arslan et al., 2006).

Most assays for animal species identification test only for husbandry species (Lahiff et al., 2001; Bottero et al., 2003; Toyoda et al., 2004, Krcmar and Rencova, 2005), and there are only a few reports for detection of pet species in commercial materials (Abdulmawjood et al., 2003; Gao et al., 2004). Although, cat, dog, and rat or mouse tissues are not commonly used as feedstuffs for other animals, their presence in these products occasionally occurs.

In this article, we describe development of a species-specific PCR method based on the 12S ribosomal RNA mitochondrial gene, for detection and identification of cat (*Felis catus*), dog (*Canis familiaris*), and rat (*Rattus norvegicus*) or mouse (*Mus musculus*) tissues in food and feedstuffs.

MATERIALS AND METHODS

All meat samples were obtained from slaughterhouses and meat-cutting installations approved ac-

[1]This study was supported by the grant Programa Vigilancia Sanitaria S-0505/AGR/000265 from the Dirección General de Universidades e Investigación, Consejería de Educación, and Comunidad de Madrid. Irene Martín and Inés López-Calleja are recipients of a fellowship from the Universidad Complutense de Madrid (Spain). Violeta Fajardo and Ularía Rojas are recipients of a fellowship from the Ministerio de Educación y Ciencia (Spain).

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Received January 19, 2007.
Accepted May 28, 2007.

Table 1. DNA sequences of the primers used in this study

Primer	Length, bp	Sequence, 5′ to 3′	Used as
12SpCATDIR	19	AATTGAATCGGGCCATGAA	Cat-specific forward primer
12SpCATINV	24	CGACTTATCTCCTCTTGTGGGTGT	Cat-specific reverse primer
12SpDOGDIR	19	AATTGAATCGGGCCATGAA	Dog-specific forward primer
12SpDOGINV	30	CTCCTCTTGTGTTTTAGTTAAGTTAATCTG	Dog-specific reverse primer
12SpRAT-MOUSEDIR	30	AAATCCAACTTATATGTGAAAATTCATTGT	Rat/mouse-specific forward primer
12SpRAT-MOUSEINV	24	TGGGTCTTAGCTATCGTCGATCAT	Rat/mouse-specific reverse primer
18SEUDIR	29	GGTAGTGACGAAAAATAACAATACAGGAC	Eukaryotes forward primer
18SEUINV	25	ATACGCTATTGGAGCTGGAATTACC	Eukaryotes reverse primer

cording to EU regulations. All procedures involving animals at the veterinary hospital were approved by the university's animal care and use committee.

Sample Selection

Meat samples from 30 individuals of each target species [cat (*Felis catus*), dog (*Canis familiaris*), rat (*Rattus norvegicus*), and mouse (*Mus musculus*)] were analyzed. These samples were obtained from the Veterinary Hospital (Facultad de Veterinaria, Universidad Complutense de Madrid, Spain). Raw meat samples from nontarget animal species were included for control purposes as follows: beef, sheep, and goat meats were provided by a local slaughterhouse; meat samples from horse, pork, rabbit, turkey, and chicken were purchased from local markets; meat samples from duck and goose were provided by Antonio de Miguel (Madrid, Spain); fish samples were purchased from local markets; samples of plant species (oats, barley, corn, rye, wheat, sunflower, rice, soybean) were purchased from local markets. All animal specimens were morphologically identified by trained veterinarians. They were transported to the laboratory under refrigeration and were processed immediately or stored frozen at –85°C until used.

Heat-treated meat samples from cat, dog, rat, and mouse were processed in autoclave in compliance with European legislation (European Commission, 2002). Three heat treatments were applied: 120°C for 50 min, 110°C for 120 min, and 133°C at 300 kPa for 20 min.

To determine the sensitivity of the assays, binary mixtures of muscle tissues in a plant matrix were prepared. Mixtures were made by adding 200 mL of sterile PBS (136 m*M* NaCl, 1.4 m*M* KH_2PO_4, 8.09 m*M* $Na_2HPO_4 \cdot 12H_2O$, 2.6 m*M* KCl, pH 7.2) to a final weight of 100 g of meat/oats components, containing 0.1, 1, 5, 10, and 25% (wt/wt) of the target species. Mixtures were homogenized using a blender (Sunbeam Oster, Delray Beach, FL). Pure samples of each target species (100%) were used as positive controls.

The effect of thermal treatments on the technique's ability to identify the target species was checked through analysis of heat-treated meat/oats mixtures. All binary mixtures were stored at –20°C until used.

Primer Design

Information obtained after alignment of 12S rRNA gene sequences available in the GenBank database, was used to design 3 specific primer pairs for the amplification of cat, dog, and rat or mouse DNA, respectively. To facilitate field application, we designed the primer pair *12SpRAT-MOUSEDIR* / *12SpRAT-MOUSEINV* to amplify with the same efficiency for rat as for mouse DNA. An additional primer pair was designed 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 (sequences are shown in Table 1). The Emboss software package version 2.2.0. and Primer Express 2.0 software (Perkin-Elmer/Applied Biosystems Division, Foster City, CA) were used for primer design.

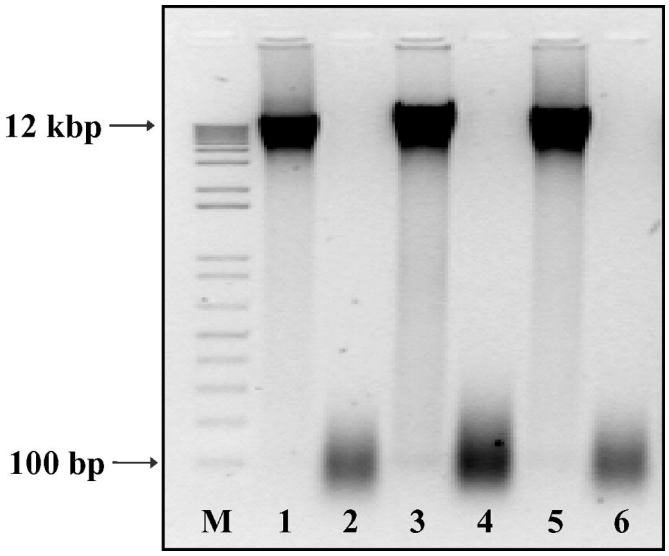

Figure 1. Gel electrophoresis of total genomic DNA extracted from raw and heat-treated (133°C, 300 kPa for 20 min) meat samples. Samples in lanes are: (1) raw cat, (2) heat-treated cat, (3) raw dog, (4) heat-treated dog, (5) raw rat, and (6) heat-treated rat (5 μL of undiluted DNA were loaded). M = molecular weight marker, 1 kb plus DNA ladder (GibcoBRL, Carlsbad, CA). Molecular sizes are indicated by the arrows.

Table 2. Specificity of the primer pairs designed for the specific detection of cat (*12SpCATDIR/12SpCATINV*), dog (*12SpDOGDIR/12SpDOGINV*), and rat or mouse (*12SpRAT-MOUSEDIR/12SpRAT-MOUSEINV*) tissues using DNA from several animal and plant species[1]

Common name	Scientific name	*12SpCATDIR* *12SpCATINV* (bp)	*12SpDOGDIR* *12SpDOGINV* (bp)	*12SpRAT-MOUSEDIR* *12SpRAT-MOUSEINV* (bp)	*18SEUDIR* *18SEUINV* (bp)
Cat	*Felis catus*	108	—[2]	—	140
Dog	*Canis familiaris*	—	101	—	140
Rat	*Rattus norvegicus*	—	—	96	140
Mouse	*Mus musculus*	—	—	96	140
Chicken	*Gallus gallus*	—	—	—	140
Turkey	*Meleagris gallipavo*	—	—	—	140
Duck	*Anas platyrhynchos* × *Cairina muschata*	—	—	—	140
Goose	*Anser anser*	—	—	—	140
Cattle	*Bos taurus*	—	—	—	140
Sheep	*Ovis aries*	—	—	—	140
Goat	*Capra hircus*	—	—	—	140
Horse	*Equus caballus*	—	—	—	140
Pig	*Sus scrofa domestica*	—	—	—	140
Rabbit	*Oryctolagus cuniculus*	—	—	—	140
Anchovy	*Engraulis encrasicolus*	—	—	—	140
Atlantic salmon	*Salmo salar*	—	—	—	140
Hake	*Merluccius* spp.	—	—	—	140
Grouper	*Epinephelus marginatus*	—	—	—	140
Nile perch	*Lates niloticus*	—	—	—	140
Monkfish	*Lophius* spp.	—	—	—	140
Rainbow trout	*Oncorhynchus mykiss*	—	—	—	140
Sardine	*Sardina pilchardus*	—	—	—	140
Sea bass	*Dicentrarchus labrax*	—	—	—	140
Sole	*Solea* spp.	—	—	—	140
Tuna	*Thunnus* spp.	—	—	—	140
Wreck fish	*Polyprion americanus*	—	—	—	140
Barley	*Hordeum vulgare*	—	—	—	140
Rice	*Oryza sativa*	—	—	—	140
Corn	*Zea mays*	—	—	—	140
Oats	*Avena sativa*	—	—	—	140
Sunflower	*Helianthus annuus*	—	—	—	140
Soybean	*Glycine max*	—	—	—	140
Rye	*Secale cereale*	—	—	—	140
Wheat	*Triticum aestiuum*	—	—	—	140

[1]*18SEUDIR*/*18SEUINV* are positive control primers.
[2]— = no amplification of the PCR product.

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 Fajardo et al. (2006). Integrity of the DNA obtained from raw and heated tissues was checked by agarose gel electrophoresis in a 1.5%, low electroendosmosis D1 agarose gel (Hispanlab S. A., Torrejon, Spain) containing 1 μg/mL of 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 of species-specific fragments was carried out in a total volume of 25 μL containing 125 ng of template DNA, 2 m*M* $MgCl_2$, 12.5 pmol of each primer, 200 μ*M* of each dNTP, and 2U 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) with the following cycling conditions: an initial heat-denaturation step at 93°C for 2 min, followed by 35 cycles consisting of 30 s at 93°C for DNA denaturation, 30 s for primer annealing, and 45 s at 72°C for DNA extension. The last extension step at 72°C was maintained for 3 min. Annealing temperature was optimized to 55°C for rat or mouse, 60°C for cat and dog, and 65°C for eukaryote primers.

The 12S rRNA amplicons (10 μL) were mixed with 2 μL of Gel Loading Solution (Sigma, Steinheim, Germany), and analyzed by electrophoresis in a 3.5% MS-8 high resolution agarose gel (Hispanlab S. A., Torrejón, Spain). Similarly, the 18S rRNA gene amplicons were electrophoresed in a 2%, low electroendosmosis D1 agarose gel (Hispanlab S. A.).

RESULTS AND DISCUSSION

There is a large body of literature for identification of economical important meats (cattle, sheep, pig, chicken) in feedstuff. However, the range of assays

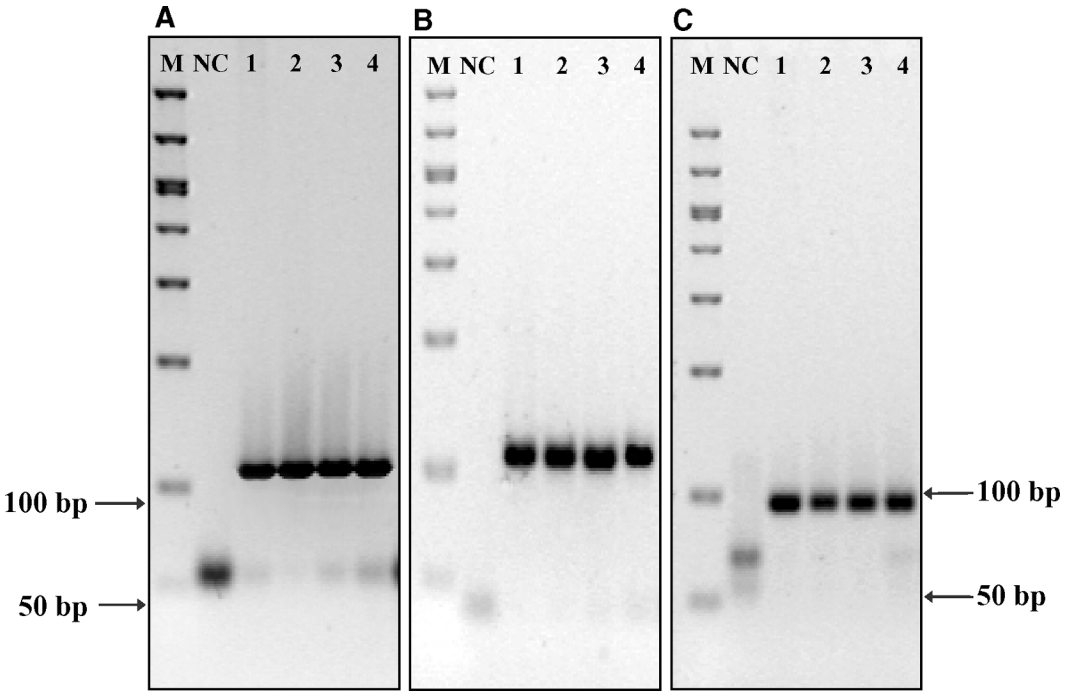

Figure 2. Electrophoretic analysis of 12S rRNA amplification products obtained using (A) cat, (B) dog, and (C) rat- or mouse-specific primers. In all electrophoretic images, lane 1 includes samples of raw muscle; and lanes 2 to 4 include samples of heat-treated muscle: 120°C for 50 min (lane 2), 110°C for 120 min (lane 3), and 133°C, 300 kPa for 20 min (lane 4). M = molecular weight marker, 50- 1,000-bp ladder (Biomarker Low, BioVentures Inc., Murfreesboro, TN); NC = negative control. The pictures are reverse images of the 3.5%, MS8 agarose gels containing ethidium bromide. Molecular sizes are indicated by the arrows.

available for detection of animal species like cat, dog, and rat or mouse, is relatively limited. In this work, a DNA-based assay using species-specific primers has been developed for detection of cat, dog, and rat or mouse tissues in food and feedstuffs.

The European Regulation concerning animal by-products not intended for human consumption provides the rules and treatments that must be used to render animal by-products (European Commission, 2002). The standard rendering condition used to produce meat and bone meal is a steam treatment at 133°C for 20 min at a pressure of 300 kPa. Such severe heat-treatment warrants denaturation of prions, but also affects DNA stability. As shown in Figure 1, total DNA extracted from heat-treated tissues (133°C, 300 kPa for 20 min) from cat (lane 2), dog (lane 4), and rat (lane 6) exhibited a typical smear pattern of nucleic acid degradation, and very low molecular weight compared with raw samples (lanes 1, 3, 5). Similar results were obtained in the analysis of raw and heated mouse DNA (data not shown). Heat degradation of DNA causes failures in PCR protocols, mainly when the fragments to be amplified are large (Matsunaga et al., 1999; Frezza et al., 2003). For this reason, amplification of DNA targets shorter than 200 bp is required for detection of animal species in heated products (Rodríguez et al., 2004).

The DNA of 32 nontarget animal and plant species were analyzed to test specificity of the primers designed. The large number of animal species that can be included in compound feedstuffs for production animals makes it necessary that any diagnostic technique should be tested for cross-reactivity against a wide range of species to avoid false positives. So, we tested specificity of the 3 primer pairs designed using DNA obtained from 24 nontarget animal species and 8 plant species. Specific DNA fragments of 108, 101, and 96 bp DNA were successfully amplified for all 30 cat, dog, rat, and mouse samples, respectively, and no cross-species amplification was observed for other animal and plant species analyzed (Table 2). Amplification patterns generated from heat-treated samples (120°C/50 min, 110°C/120 min, or 133°C/300 kPa/20 min) resembled those obtained for raw samples (Figure 2).

Most food or feed products to be tested would not be expected to be contaminated with cat, dog, rat, or mouse tissues, and they would not produce an amplification product with these species-specific primers. Thus, in order to avoid false-negative results due to a failure of the amplification procedure, it is essential to include reference samples of the target species as positive controls together with the DNA from unknown samples. Also, to facilitate diagnostic field application, we designed a positive control primer pair, *18SEUDIR* and *18SEUINV*, that should amplify a conserved region of 140 bp of the 18S rRNA gene in all the plant and animal species tested (Table 2).

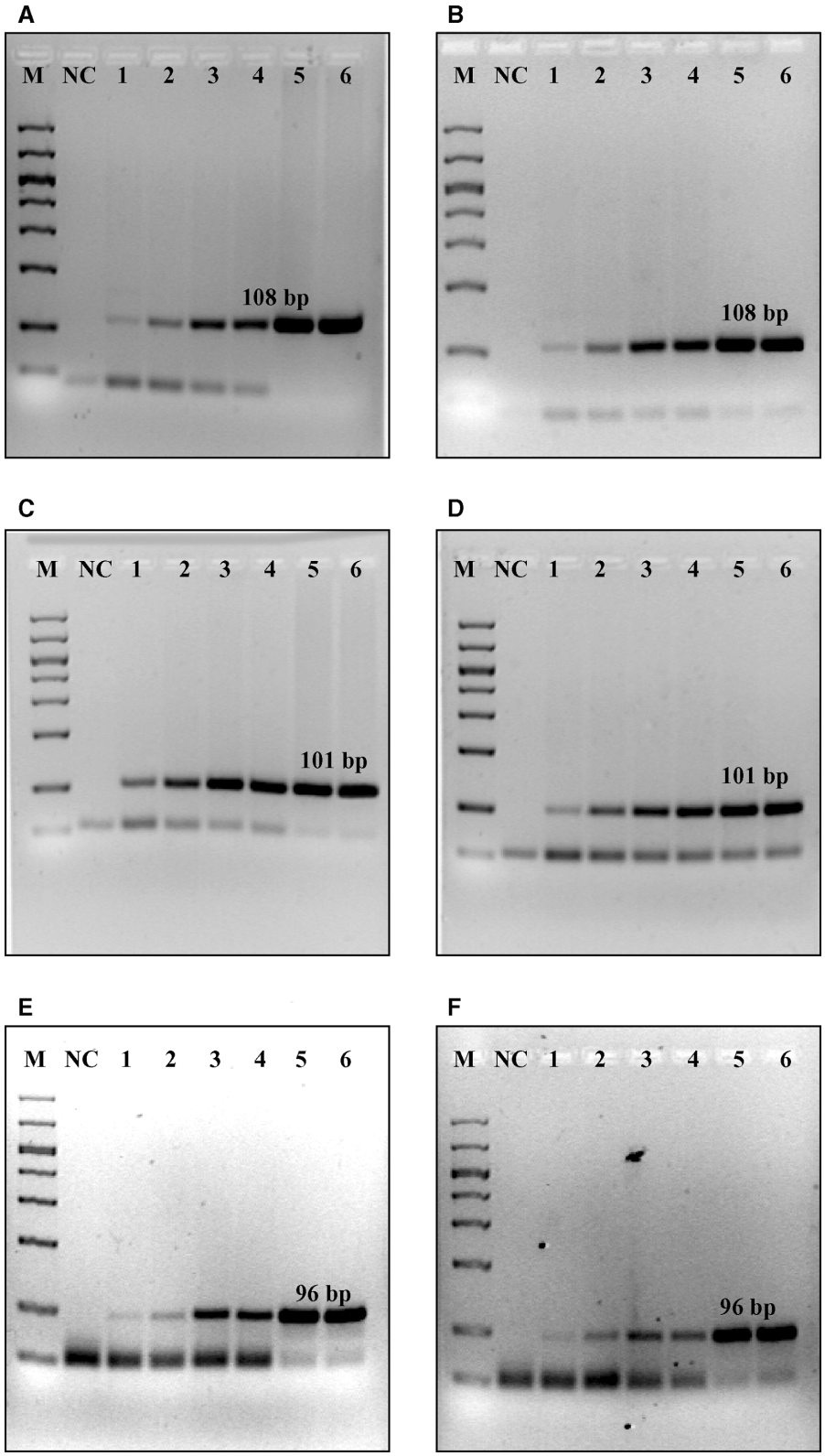

Figure 3. Electrophoretic analysis of the 12S rRNA PCR products obtained from raw and heat-treated (133°C, 300 kPa for 20 min) meat/oats mixtures, using cat, dog, and rat or mouse species-specific primers. Lanes 1 to 6 are samples of binary mixtures containing 0.1, 1, 5, 10, 25, and 100% of the target species, respectively. (A) raw cat meat in oats; (B) heat-treated cat meat in oats; (C) raw dog meat in oats; (D) heat-treated dog meat in oats; (E) raw rat meat in oats; and (F) heat-treated rat meat in oats. Similar results were obtained for raw and heat-treated mouse/oats mixtures using the rat- or mouse-specific primers (results not shown). M = molecular weight marker, 50- 1,000-bp ladder (Biomarker Low, BioVentures Inc., Murfreesboro, TN); NC = negative control. The pictures are reverse images of the 3.5%, MS8 agarose gels containing ethidium bromide. Molecular sizes are indicated.

The sensitivity of cat-, dog-, and rat- or mouse-specific assays (lower amount of the target species 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% (wt/wt) of the target species. With a lower percentage of target species (cat, dog, rat, or mouse) in the admixture, a fainter band was obtained in the PCR with species-specific primers. The lower percentage producing visible DNA amplification using the primer pairs *12SpCATDIR/12SpCATINV*, *12SpDOGDIR/12SpDOGINV*, and *12SpRAT-MOUSEDIR/12SpRAT-MOUSEINV* was 0.1% for raw meat/oats mixtures (Figure 3, panels A, C, and E). The steam treatment at 133°C for 20 min at 300 kPa applied to cat/oats mixtures (Figure 3B), dog/oats mixtures (Figure 3D), and rat/oats mixtures (Figures 3F) did not modify the detection limit. Similar results were obtained for mouse/oats mixtures using rat- or mouse-specific primers (results not shown).

According to the results obtained with PCR, using specific primers facilitates detection of cat, dog, rat, and mouse tissues in food and feed with a high sensitivity and specificity. This method could be a useful tool for detection of undeclared ingredients in foods and feeds, in order to enforce labeling regulations and compliance of commercial products with feed bans.

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