Technical note: Detection of chicken, turkey, duck, and goose tissues in feedstuffs using species-specific polymerase chain reaction

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J ANIM SCI 2007, 85:452-458.
doi: 10.2527/jas.2006-350

Technical note: Detection of chicken, turkey, duck, and goose tissues in feedstuffs using species-specific polymerase chain reaction[1]

I. Martín, T. García,[2] V. Fajardo, I. López-Calleja, M. Rojas, M. A. Pavón, P. E. Hernández, I. González, and R. Martín

Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain

ABSTRACT: PCR method was applied for the qualitative identification of chicken (*Gallus gallus*), turkey (*Meleagris gallipavo*), duck (*Anas platyrhynchos* × *Cairina muschata*), and goose (*Anser anser*) tissues in feedstuffs, on an individual basis. The assay uses oligonucleotide primers that are specific for each avian species, targeting the 12S rRNA mitochondrial gene. The primers designed generated amplicons of 95, 122, 64, and 98 bp length for chicken, turkey, duck, and goose, respectively. The specificity of the primers was tested against 29 animal species including mammals, birds, and fish, as well as 8 plant species. Analysis of experimental feedstuffs demonstrated the detection of each target species in the range of 0.1 to 100%. The performance of this 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 for the accurate identification of tissues from these 4 avian species in products submitted to denaturing technologies, for which other methods cannot be applied.

Key words: chicken, duck, goose, species-specific polymerase chain reaction, turkey, 12S ribosomal ribonucleic acid gene

J. Anim. Sci. 2007. 85:452–458
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INTRODUCTION

Identification of tissues from various species in feed products has focused on the prevention of fraud and assessment of the safety risk derived from introduction of any animal material that might be harmful to human or animal health (Pascoal et al., 2004). In response to bovine spongiform encephalopathy, many nations have implemented regulations prohibiting feeding of mammalian materials to ruminants (European Commission, 2002). In North America, materials from equine, porcine, and nonmammalian species such as fish and poultry are permitted in ruminant feeds. Thus, it is important to identify any animal species that might be present in ruminant feeds. Most studies have focused on 4 livestock species (cattle, pig, sheep, goat), and only a few reports have dealt with birds (chicken, turkey). Fewer studies reported work related to other birds such as goose and duck.

Currently, the official method for determination of prohibited materials in animal feed is microscopy (European Commission, 1998). However, microscopy does not allow for determination of species origin of the material and is time-consuming and labor-intensive (Armour and Blais, 2006). Several analytical approaches have been made to identify animal species in a wide array of degraded and processed substrates, broadly based on detecting protein or DNA (Aida et al., 2005). However, proteins are denatured during heat and pressure processing, making species detection in a processed sample more difficult. Polymerase chain reaction allows direct species identification, but high temperatures involved in rendering animal products (133°C) cause fragmentation of DNA (Frezza et al., 2003), making it necessary to develop PCR assays that rely on amplification of short DNA targets (Krcmar and Rencova, 2005).

In this paper, we describe a species-specific PCR method for detection and identification of chicken, turkey, duck, and goose tissues in feedstuffs, based on selective PCR amplification of short DNA fragments in the 12S ribosomal RNA mitochondrial gene.

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

[2]Corresponding author: tgarcia@vet.ucm.es

Received June 1, 2006.
Accepted September 5, 2006.

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

Primer	Length, bp	Sequence (5′ to 3′)	Used as
12SpCHICKENDIR	26	ACATAGAACAAACGAAAAAGGATGTG	Chicken specific forward primer
12SpCHICKENINV	23	CGTCTTAAAGTGAGCTTAGGGCG	Chicken specific reverse primer
12SpTURKEYDIR	26	CCACCTAGAGGAGCCTGTTCTRTAAT	Turkey specific forward primer
12SpTURKEYINV	27	TTGAGCTCACTATTGATCTTTCAGTTT	Turkey specific reverse primer
12SpDUCKDIR	24	CATAATTAATACCCTGTAAATGCC	Duck specific forward primer
12SpDUCKINV	25	TCCAGTATGCTTACCTTGTTACGAC	Duck specific reverse primer
12SpGOOSEDIR	24	TCAAGGTATAGCCTATGGAGTCGA	Goose specific forward primer
12SpGOOSEINV	22	CTAAATCCGCCTTCCAGAAATG	Goose specific reverse primer
FWnad1	22	AGGCAAGAACCATGCTTTCAGA	Plant forward primer
REVnad1	19	CAGCCCTTTCCTCCTGCCG	Plant reverse primer

MATERIALS AND METHODS

All meat samples were obtained from slaughterhouses and meat-cutting installations approved according to European Union regulations. All procedures involving animals at the veterinary hospital were approved by the university's animal care and use committee (Comité de Experimentación Animal, Universidad Complutense de Madrid).

Sample Selection

Whole duck and goose specimens were provided by Antonio de Miguel (Madrid, Spain). Raw beef, sheep, and goat muscles were provided by a local slaughterhouse. Raw meat samples from horse, pork, rabbit, turkey, and chicken were purchased from local markets. Muscle samples of cat, dog, and rat tissues were obtained from The Veterinary Hospital (Facultad de Veterinaria, Universidad Complutense de Madrid, Spain). Muscle samples from red deer, fallow deer, roe deer, and chamois were obtained from several Spanish meat-cutting installations. Fish samples were purchased from local markets.

All animal specimens were morphologically identified by trained veterinarians. They were transported to the laboratory under refrigeration, and processed immediately or stored frozen at –85°C until used.

Heat-treated muscle samples from chicken, turkey, duck, and goose were processed in an autoclave in compliance with European legislation (European Commission, 2002). Three different heat-treatments were applied: 120°C for 50 min, 110°C for 120 min, and 133°C at 300 kPa for 20 min.

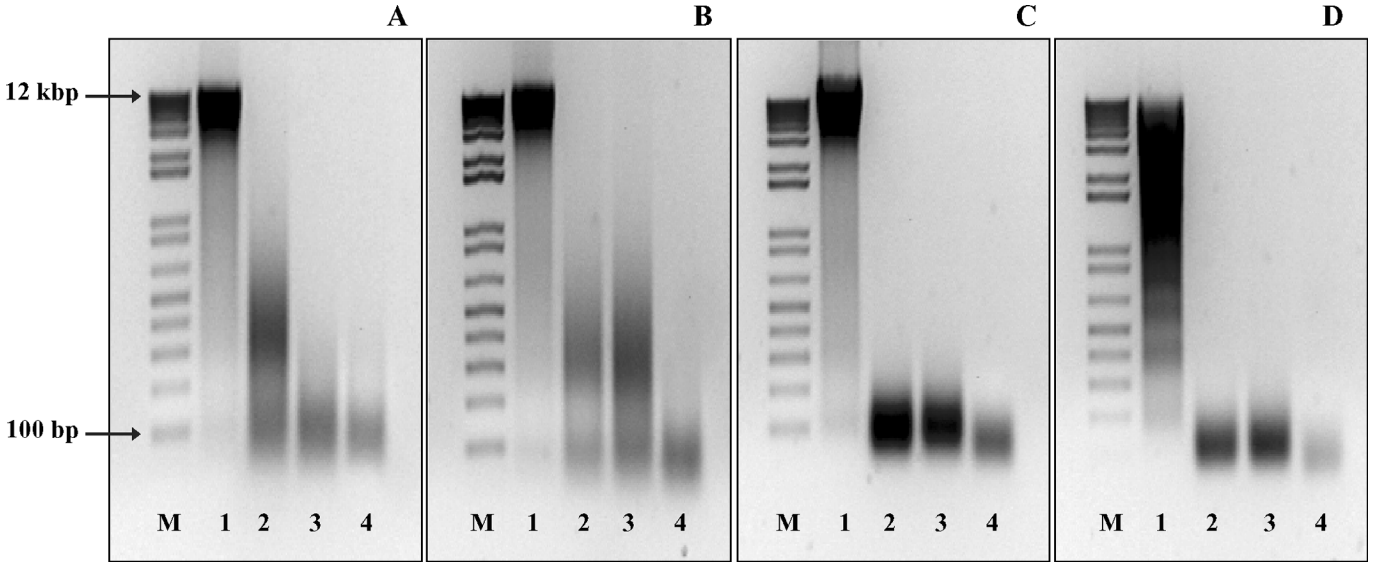

Figure 1. Gel electrophoresis of total genomic DNA extracted from (A) chicken, (B) turkey, (C) duck, and (D) goose tissues. Lanes 1 to 4 include samples of raw muscle (lane 1) and heat-treated muscle (lane 2, 120°C for 50 min; lane 3, 110°C for 120 min; and lane 4, 133°C, 300 kPa for 20 min). Five microliters of the total DNA extracted 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 chicken (*12SpCHICKENDIR/12SpCHICKENINV*), turkey (*12SpTURKEYDIR/12SpTURKEYINV*), duck (*12SpDUCKDIR/12SpDUCKINV*), and goose (*12SpGOOSEDIR/12SpGOOSEINV*) tissues, using DNA from several animal and plant species

Common name	Scientific name	*12SpCHICKENDIR* *12SpCHICKENINV*	*12SpTURKEYDIR* *12SpTURKEYINV*	*12SpDUCKDIR* *12SpDUCKINV*	*12SpGOOSEDIR* *12SpGOOSENV*
Chicken	*Gallus gallus*	95 bp	—[1]	—	—
Turkey	*Meleagris gallipavo*	—	122 bp	—	—
Duck	*Anas platyrhynchos* × *Cairina muschata*	—	—	64 bp	—
Goose	*Anser anser*	—	—	—	98 bp
Cattle	*Bos taurus*	—	—	—	—
Sheep	*Ovis aries*	—	—	—	—
Goat	*Capra hircus*	—	—	—	—
Chamois	*Rupicapra rupicapra*	—	—	—	—
Fallow deer	*Dama dama*	—	—	—	—
Roe deer	*Capreolus capreolus*	—	—	—	—
Red deer	*Cervus elaphus*	—	—	—	—
Horse	*Equus caballus*	—	—	—	—
Pig	*Sus scrofa domestica*	—	—	—	—
Rabbit	*Oryctolagus cuniculus*	—	—	—	—
Cat	*Felis catus*	—	—	—	—
Dog	*Canis familiaris*	—	—	—	—
Rat	*Rattus norvegicus*	—	—	—	—
Anchovy	*Engraulis encrasicolus*	—	—	—	—
Atlantic salmon	*Salmo salar*	—	—	—	—
Hake	*Merluccius* spp.	—	—	—	—
Grouper	*Epinephelus marginatus*	—	—	—	—
Nile perch	*Lates niloticus*	—	—	—	—
Monkfish	*Lophius* spp.	—	—	—	—
Rainbow trout	*Oncorhynchus mykiss*	—	—	—	—
Sardine	*Sardina pilchardus*	—	—	—	—
Sea bass	*Dicentrarchus labrax*	—	—	—	—
Sole	*Solea* spp.	—	—	—	—
Tuna	*Thunnus* spp.	—	—	—	—
Wreck fish	*Polyprion americanus*	—	—	—	—
Barley	*Hordeum vulgare*	—	—	—	—
Rice	*Oryza sativa*	—	—	—	—
Corn	*Zea mays*	—	—	—	—
Oats	*Avena sativa*	—	—	—	—
Sunflower	*Helianthus annuus*	—	—	—	—
Soybean	*Glycine max*	—	—	—	—
Rye	*Secale cereale*	—	—	—	—
Wheat	*Triticum aestiuum*	—	—	—	—

[1]—No amplification of the PCR product.

Samples of plant species (oats, barley, corn, rye, wheat, sunflower, rice, soybean) were purchased from local markets.

To evaluate the sensitivity of the test, binary mixtures of muscular 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 the meat/oats components containing 0.1, 1, 5, 10, 25, and 100% (wt/wt) of the target species and homogenizing the mixture using a blender (Sunbeam Oster, Delray Beach, FL). Pure samples of the target species were used as positive controls.

The effect of thermal treatments on the sensitivity of the technique for the target species was checked through analysis of binary mixtures prepared as described above but using the different percentages of heat-treated animal tissues. All binary mixtures were stored at –20°C until used.

Primer Design

Four species-specific primer pairs were designed for amplification of avian DNA at the *12S rRNA* gene, and an additional primer pair was designed to amplify a conserved region of 250 bp of the *nad1* gene in all plant species commonly used in feedstuffs (sequences shown in Table 1). These sets of primers were designed based on various animal and plant species sequences available in the GenBank database, such as wheat (accession number, X57967), rice (DQ143138), soybean (AJ428875), barley (AF521177), corn (AY705912), rat (NC001665), and pig (DQ534707). 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.

PCR Amplification

Genomic DNA was obtained from 200 mg of animal, plant, or binary mixture materials, using a Wizard DNA

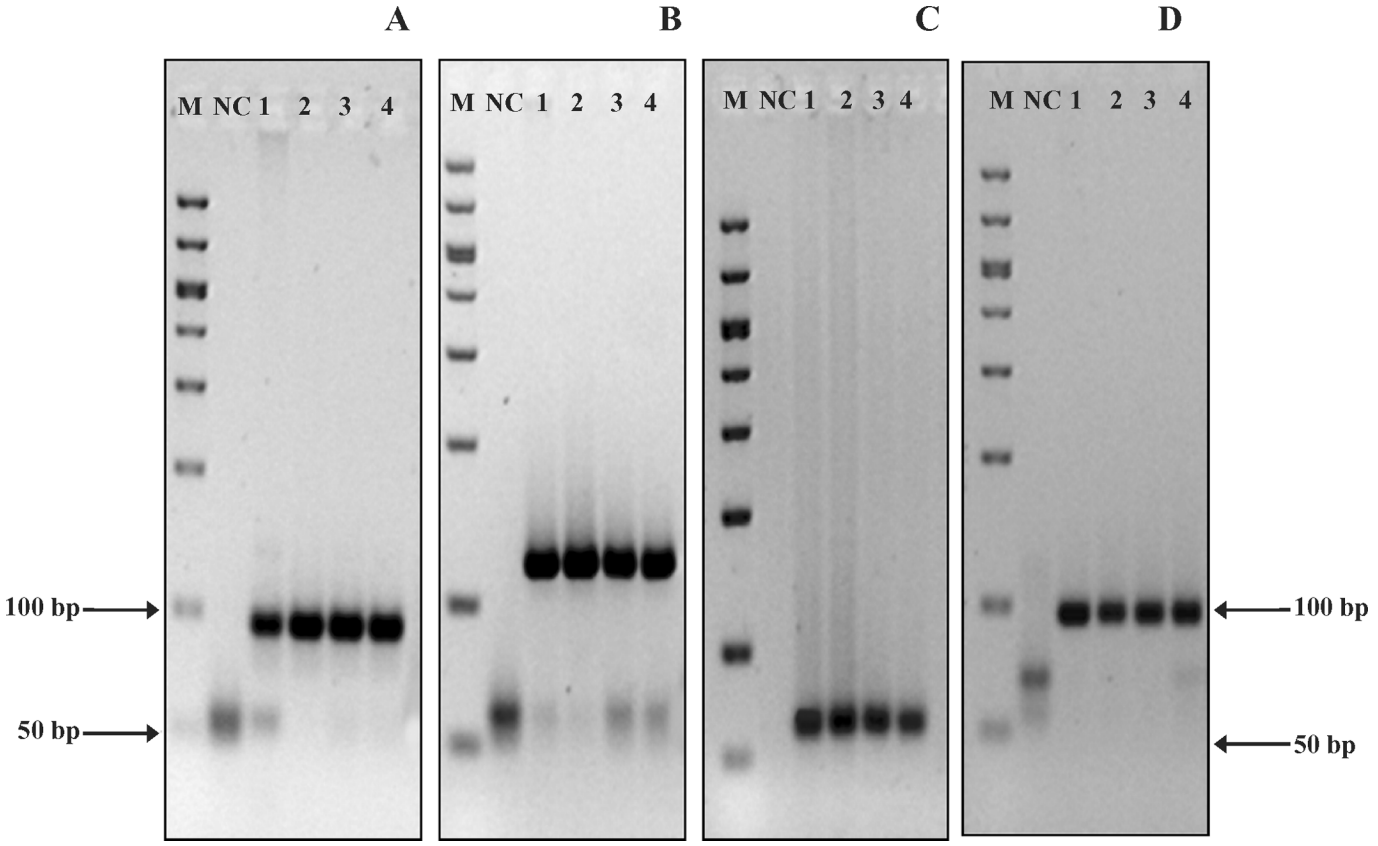

Figure 2. Electrophoretic analysis of 12S rRNA amplification products obtained using (A) chicken-, (B) turkey-, (C) duck-, and (D) goose-specific primers. In all electrophoretic images, lane 1 includes samples of raw muscle, and lanes 2 to 4 include samples of heat-treated muscle (lane 2, 120°C for 50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

Clean-up System kit (Promega Corp., Madison, WI) as described by Fajardo et al. (2006). Integrity of the DNA 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 of ethidium bromide/mL 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 50 μL containing 250 ng of template DNA, 2 m*M* $MgCl_2$, 25 pmol of each primer, 200 μ*M* of each dNTP, and 2 U 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 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 62°C for chicken, 63°C for turkey, 55°C for duck, 60°C for goose, and 62°C for plant-specific 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.). Similarly, the *nad1* gene amplicons were electrophoresed in a 2%, low electroendosmosis D1 agarose gel (Hispanlab S. A.).

RESULTS AND DISCUSSION

To check the influence of processing treatments on suitability of the PCR method developed herein, we evaluated by agarose gel electrophoresis the amounts and degradation levels of total DNA obtained from heat-treated samples. Total DNA extracted from heat-treated tissues from chicken, turkey, duck, and goose exhibited a typical smear pattern of nucleic acid degradation and very low weight compared with the raw samples (Figure 1). For this reason, primers targeting DNA fragments shorter than 200 bp are preferable in heat-treated products (Rodríguez et al., 2004).

The specificity of the 4 species-specific primer pairs designed was tested using DNA obtained from muscle

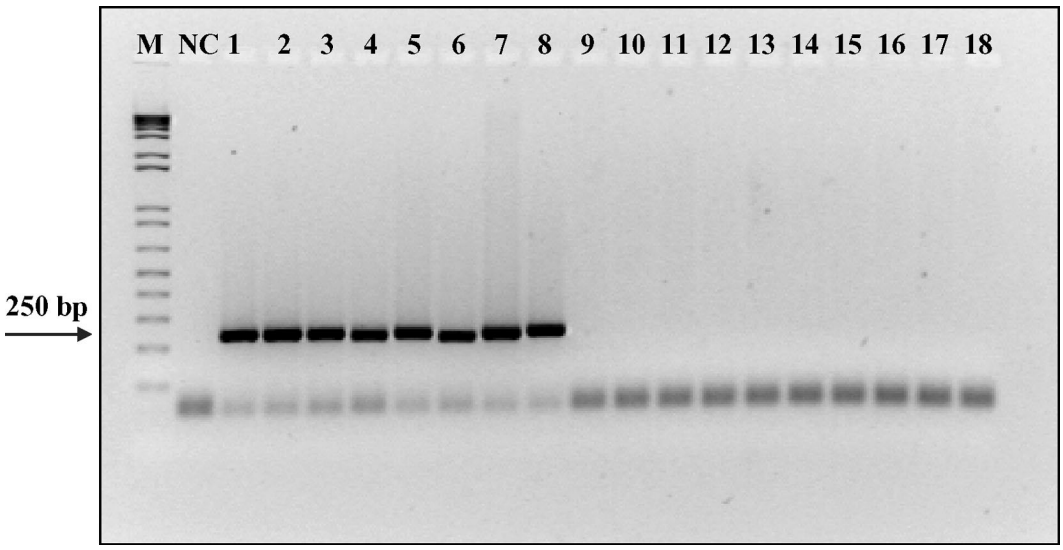

Figure 3. Electrophoretic analysis of the plant-specific *nad1* PCR products obtained with primers FWnad1 and REVnad1 from (lane 1) oats, (lane 2) barley, (lane 3) corn, (lane 4) rye, (lane 5) wheat, (lane 6) sunflower, (lane 7) soybean, (lane 8) rice, (lane 9) beef, (lane 10) sheep, (lane 11) goat, (lane 12) pig, (lane 13) rabbit, (lane 14) horse, (lane 15) goose, (lane 16) chicken, (lane 17) duck, and (lane 18) turkey tissues. M = Molecular weight marker, 1 kb plus DNA ladder (GibcoBRL, Carlsbad, CA); NC = negative control. Molecular size is indicated by the arrow. The picture is a reverse image of the agarose gel containing ethidium bromide.

samples of 29 animal species (13 mammals, 12 fish, 4 birds) and 8 plant species, and no cross-species amplification was observed (Table 2). Demonstration of specificity against a large number of species is very important because many animal materials can be included in compound feedstuffs for animal production. When a reduced number of species are used to assess specificity of the assay, there is always a risk of finding cross-reactivity with not tested close-related species, limiting the value of the assay for routine analysis.

Specific DNA fragment of 95, 122, 64, and 98 bp DNA were successfully amplified with chicken, turkey, duck, and goose primer sets, respectively, whereas no homologous product was amplified from any other animal or plant DNA tested (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).

Because many food or feed products to be tested would be not contaminated with avian DNA, they would not produce an amplification product with avian-specific primers. Thus, it is essential to include positive controls in order to avoid false-negative results due to the failure of the amplification procedure. To facilitate diagnostic field application, we designed the positive control primers *FWnad1* and *REVnad1* that should amplify a conserved region of 250 bp of the *nad1* gene in all the plant species commonly used in feedstuffs. Amplification of a PCR fragment of approximately 250 bp was obtained from all the plant species but not from any of the animal species tested (Figure 3). The mitochondrial encoded gene *nad1* was selected in this work because it has an acceptable length and an adequate grade of mutation and because there are sequences of several plant species available in the databases (Herman et al., 2003).

To determine the lower percentage producing visible DNA amplification of the assay for the species analyzed, PCR amplification was performed on binary mixtures containing 0.1, 1, 5, 10, 25, and 100% (wt/wt) of the target species. For all species, the lower the percentage of target species in the admixture, the fainter the band obtained in the PCR with the species-specific primers. The lower percentage producing visible DNA amplification using chicken, turkey, duck, and goose specific primer pairs was 0.1% for binary mixtures submitted to 133°C/300 kPa/20 min (Figure 4). Similar results were obtained from raw or heat-treated (120°C/50 min and 110°C/120 min) binary mixtures (results not shown).

Results of the present work demonstrate that species-specific PCR is a useful method to test the presence of low levels (0.1%) of avian ingredients in feedstuffs, even for samples that were subjected to severe heat-treatment, for which other methods cannot be applied. The method is quick and sensitive, avoiding cross-amplifications of other animal and plant species. However, a problem of any analytical method based on DNA analysis is that species-specific PCR amplifies and detects avian DNA in feedstuffs, regardless of the tissue. Thus, because eggs also contain avian DNA, it will be amplified and detected by PCR. The European Commission (2003) prohibits feeding animal protein to ruminants, but milk, milk-based products and colostrum, eggs, egg

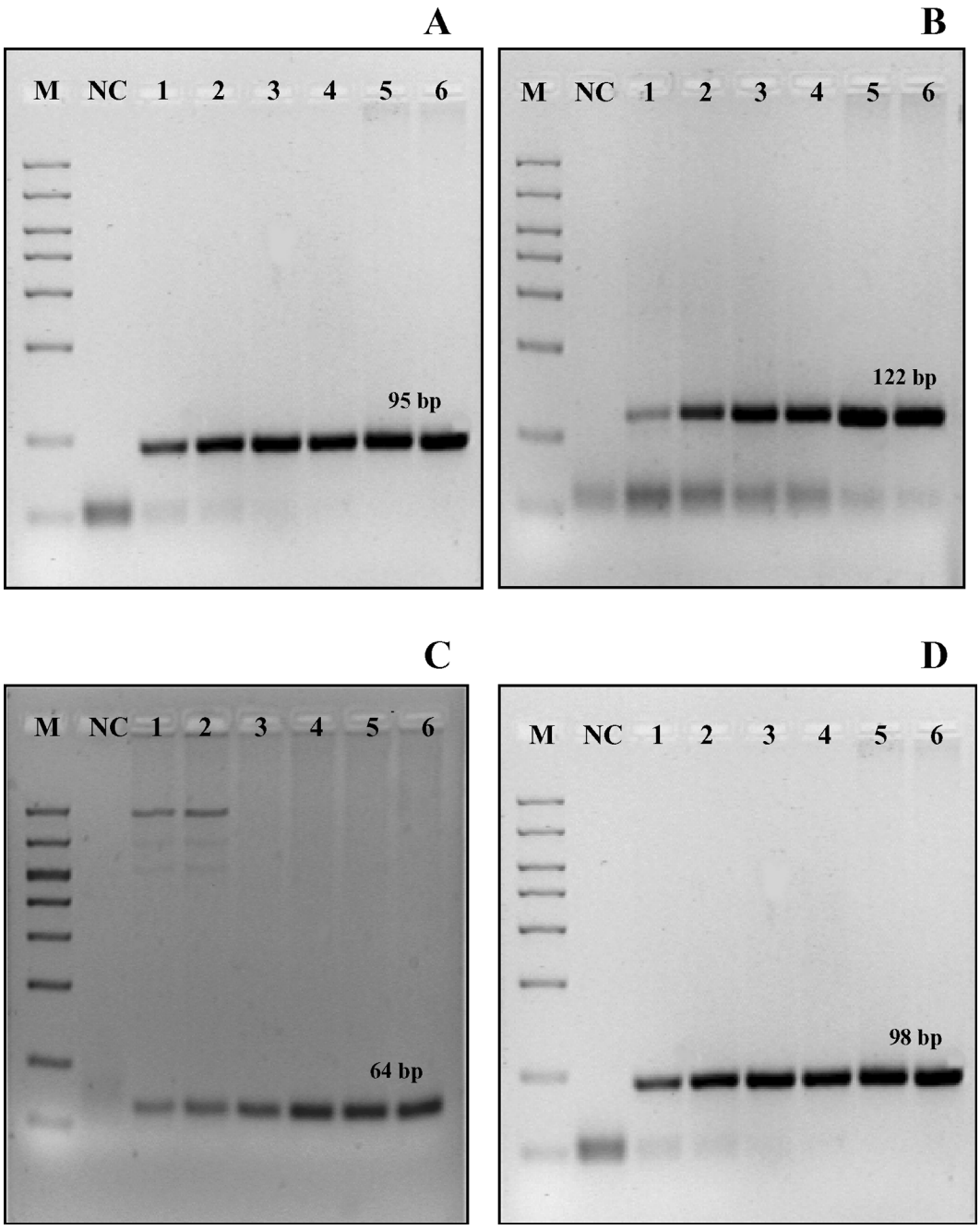

Figure 4. Electrophoretic analysis of the 12S rRNA PCR products obtained from binary mixtures of heat-treated (133°C, 300 kPa, and 20 min) tissues from avian species in oats, using species-specific primers for (A) chicken, (B) turkey, (C) duck, and (D) goose. Lanes 1 to 6 are samples of binary mixtures containing 0.1, 1, 5, 10, 25, and 100% of the target species, respectively. M = Molecular weight marker, 50–1,000 bp ladder (Biomarker Low, BioVentures, Murfreesboro, TN); NC = negative control. Molecular sizes are indicated. The pictures are reverse images of the 3.5% MS8 agarose gels containing ethidium bromide.

products, and gelatin derived from nonruminants are exempt from this regulation.

Compared with alternative techniques for species identification such as PCR-RFLP (Pascoal et al., 2004) or DNA sequencing (Colombo et al., 2002), PCR using specific primers offers the advantages of being less expensive and more useful for routine analysis of large numbers of samples.

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