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Beef- and Bovine-Derived Material Identification in Processed and Unprocessed Food and Feed by PCR Amplification

JORGE H. CALVO,[†] CLEMENTINA RODELLAR, PILAR ZARAGOZA,* AND ROSARIO OSTA

Laboratorio de Genética Bioquímica, Facultad de Veterinaria, C/Miguel Servet 177, 50013 Zaragoza, Spain

This research developed and evaluated a PCR procedure to detect beef in heated and unheated meat, sausages, and canned food, using a specific and sensitive method. To confirm the effectiveness and specificity of this fragment, we tested 45 cattle blood DNA samples (from different breeds) and obtained positive results. With 125 samples tested from other species, the specific beef amplification was not detected. Feed components intended for cattle nutrition were also checked, and bovine-derived material was detected. Using this method we can detect the degree of contamination up to 0.01% raw beef in pork. In the same way, 1% beef was detected in cooked meat mixtures and bovine-derived material in concentrate mixtures. Beef has been identified in both heated and unheated meat products, sausages, canned food, and hamburgers. In conclusion, specific PCR amplification of a repetitive DNA element seems to be a powerful technique for the identification of beef in processed and unprocessed food, because of its simplicity, specificity and sensitivity. Furthermore, feed components intended for cattle nutrition can be checked. The procedure is also much cheaper than other methods based on RFLPs-PCR, immunodiffusion, and other techniques that need expensive equipment.

KEYWORDS: Cattle; food misinformation; species; feed; beef

INTRODUCTION

There have been a tremendous growth in quality product consumption and a change in attitudes in this respect. Nowadays, consumers demand quality products that are well labeled. However, fraudulent or unintentional mislabeling still exits and may not be detected. Furthermore, some population groups do not desire beef because of the menace of transmission of bovine spongiform encephalopathy (*1*). This population demand methods to detect beef in food. Therefore, feed components intended for cattle nutrition must be checked for the presence of bovine-derived materials.

Numerous analytical methods that rely on proteins analysis have been developed for beef identification such as electrophoresis techniques (*2*, *3*), liquid chromatography (*4*), and immunoassays (*5*). However, proteins lose their biological activity after an animal's death, and their presence and characteristics depend on cell types. Furthermore, most of them are heat-labile. Thus, molecular biological methods allow the demonstration of DNA also in heat-treated material and are, therefore, suitable for the identification of species-specific DNA in meat and bone meal and concentrate mixtures in general.

The dot-blot technique was the first genetic approach for determination of species identity (*6*). At present, however, polymerase chain reaction (PCR) is the technique of choice for species identification (*7*). Some PCR approaches are random-amplified polymorphic DNA fingerprints-PCR (RAPD-PCR) (*8*), DNA mithochondrial D-loop analysis (*9*), and restriction fragments length polymorphic (RFLPs) analysis of different PCR fragments (*10*, *11*, *12*). Detection of bovine-derived material in compound feeds has also been performed by PCR analysis (*13*, *14*). In this work we have focused on evaluating a PCR procedure to detect cattle in processed and unprocessed food and feed.

MATERIAL AND METHODS

DNA Extraction and Sample Selection. Meat samples were taken, minced with a pair of scissors, and placed into a 1.5 mL tube to avoid contamination. Samples containing 0%, 0.001%, 0.005%, 0.01%, 0.1%, 1%, 2%, 5%, 10%, 25%, 50%, 75%, and 100% beef in pork were taken, each with a total weight of 0.5 g. Meat samples were heated, autoclaving at 80 and 120 °C, using a holding period of 30 min. Raw samples were also analyzed. In the same way, bovine-derived material (meat- and bone-meal heated at 120 °C for 30 min) in concentrate feed mixtures were made containing 0%, 0.001%, 0.005%, 0.01%, 0.1%, 1%, 2%, 5%, 10%, 25%, 50%, 75%, and 100% bovine-derived material, each weighing 0.5 g.

Other food products and raw meat from other species were also analyzed to verify whether they contained bovine DNA (**Table 2**). The analyzed beef products were bought in a store in order to verify the beef content. Canned beef products were labeled as sterilized products. Genomic DNA was extracted according to a previously described

* Corresponding author. Fax: 34976761612. E-mail: pilartar@posta.unitar.es.

[†] Present address: Departamento de Mejora Genética, INIA, Ctra. La Coruña Km 7.5, 28040 Madrid, Spain. Phone: 34 913473591. Fax: 34913572293. E-mail: jhcalvo@inia.es.

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Table 1. Specificity of the Technique to Blood Samples from a Variety of Species

species	*n*
cattle[a]	45
pigs	20
sheep	20
goat	5
deer	10
chicken	10
turkey	3
duck	5
rabbits	5
horses	20
dogs	20
humans	3
rat	2

[a] Normanda, Pirenaica, Murciana, Menorquina, Asturiana de las Montañas, Asturiana de los Valles, Mallorquina, Holstein, and Bullfighting breeds (n=5/breed).

Table 2. Food Products Analyzed and Results for Specific Beef PCR Amplification

sausages	
pork (n = 5)	−
meat mixtures (n = 2)	+[a]
salami-type sausage (n = 1)	+
pork ham (n = 2)	−
pork salt ham, chorizo (n = 1 each)	−
duck ham (n = 1)	−
hamburgers	
beef (n = 5), pork and beef (n = 2)	+
pork (n = 2), pork and chicken (n = 2)	−
chicken (n = 5), turkey (n = 1)	−
canned beef products (n = 3)	+
raw meat	
pork (n = 5)	−
duck, turkey, chicken, rabbit (n = 2 each)	−
beef (n = 5)	+
lamb (n = 1)	−

[a] Beef detection by 84 bp amplification.

procedure (*15*). Five hundred milligrams were incubated, adding 0.5 mL of extraction buffer (10 mM Tris (pH 8.0), 100 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate (SDS)), and 100 mg of proteinase K. The sample was incubated at 52 °C for 2h. Protein was precipitated by addition of 200 mL of satured NaCl (0.5 M) followed by agitation and centrifugation (7000*g*) for 15 min at 4 °C. The supernatant was subjected to phenol-chloroform extraction. Two volumes of cold ethanol were added, and the solution was kept at −80 °C for 1 h. The resulting DNA precipitated was collected by centrifugation (7000*g*) for 30 min at 4 °C and then washed in 70% ethanol, vacuum-dried, and resuspended in 30 mL of buffer [Tris (10 mM)−EDTA (1.0 mM), pH 7.5].

Specificity. To test the specificity of the technique, we analyzed 170 unrelated blood samples from several species and breeds of cattle (**Table 1**). Genomic DNA was extracted from blood according to a previously described procedure (*16*). White blood cells were obtained centrifuging (1500*g* for 10 min) 3 mL of blood mixed with 5 mL of TKM1 solution (10mM TRIS−HCl pH 8.0, 10 mM KCl, 10 mM MgCl2, 2 mM EDTA) and 2% (w/v) Triton X-100. White blood cells at the bottom of the tube were incubated with 0.8 mL of TKM2 solution (10mM TRIS−HCl pH 8.0, 10 mM KCl, 10 mM MgCl2, 2 mM EDTA, 4mM NaCl) and 50 mL of 10% (w/v) sodium dodecyl sulfate at 55 °C for 10 min. Protein was precipitated by addition of 300 mL of satured NaCl (6 M) followed by agitation and centrifugation (11000*g*) for 5 min at 4 °C. Two volumes of cold ethanol were added, and the solution was kept at −80 °C for 1 h. The resulting DNA precipitated was collected by centrifugation (7000*g*) for 30 min at 4 °C and then washed in 70% ethanol, vacuum-dried, and resuspended in 30 mL of buffer [Tris (10 mM)−EDTA (1.0 mM), pH 7.5].

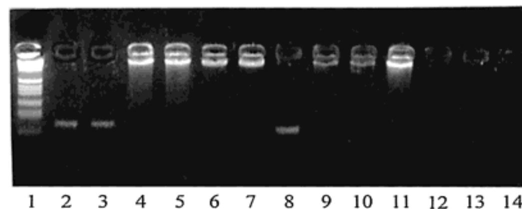

Figure 1. 84 bp PCR amplification. Line 1, BRL 1Kb marker (Gibco); lines 2, 3, and 8, cattle; line 4, goat; line 5, sheep; line 6, chicken; line 7, turkey; line 9, pig; line 10, deer; line 11, rabbit; line 12, horse; line 13, rabbit; line 14, negative control.

PCR Amplification of Specific Fragment Of Bovine DNA. The set of primers used for PCR-specific cattle amplification was chosen from the 1709 satellite DNA (GenBanK accession no. X00979). This satellite DNA was highly repeated in bovine genome[17]. The primers were designed as follows: 5′-CAGAACTTGAATTTATTTG-3′ (forward primer) and 5′-GTGACGACAGTGTACTGTTC-3′ (reverse primer).

Double-stranded amplifications were carried out in a final volume of 25 μL, containing 10 mM Tris-HCl, pH 8.8,1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 20 pmol of each primer, 30 ng of template DNA, and 2U of Taq polymerase (Promega, Promega Corporation, Madison, WI 53711-5399). The DNA was amplified in a Biometra Thermal cycler (Biometra Ltd., Whatman House, St. Leonard's Road, 20/20 Maidstone, Kent, ME 16 OLS, UK). Thirty-five cycles were performed with the following step-cycle profile: strand denaturation at 94 °C for 30 s, primer annealing at 53 °C for 30 s, and primer extension at 72 °C for 30s. The last extension step was 5 min longer. An initial denaturation at 94 °C for 4 min was performed to improve the final result. Electrophoresis of a 10μL portion of the amplification was carried out for 45 min at 100 V in a 2% agarose gel, containing ethidium bromide (1μg/mL) in TBE buffer. DNA fragment was visualized by UV transillumination.

Sensitivity. Mixtures of beef and pork raw and heated were made to detect the minimum quantity of DNA. Samples containing 0%, 0.001%, 0.005%, 0.01%, 0.1%, 1%, 2%, 5%, 10%, 25%, 50%, 75%, and 100% beef in pork were taken. Furthermore, bovine-derived material (meat- and bone-meal heated at 120 °C for 30 min) in concentrate feed mixtures were analyzed. For this purpose, a PCR was carried out under the same PCR conditions as above.

RESULTS AND DISCUSSION

The objective of the experiment was to test the applicability of PCR as a routine method for the detection of beef- and bovine-derived material. Oligonucleotides used as primers for bovine-specific PCR were based on the 1709 bovine satellite. A 84 bp amplification was obtained (**Figure 1**). To confirm the effectiveness and the specificity of this fragment, it was tested in 45 cattle blood DNA, from eight different breeds, obtaining the 84 bp specific cattle band. With 125 samples tested from other species the positive amplification was not obtained (**Table 1**).

The total amount of DNA and the percentage of beef in the samples *is important data for verifying* the sensitivity of the method. In this respect, Meyer et al. (*10*) detected beef in heated beef mixtures at levels below 1% with a fragment of the cytochrome *c* cDNA, and Matsunaga et al. (*12*) detected 250 pg of beef DNA, in DNA mixtures. In raw pork and beef mixtures, we detected up to 0.01% beef in pork (**Figure 2**). This percentage corresponds to 2.5 pg of bovine DNA. In cooked meat mixtures, 1% beef was detected (**Table 3** and **Figure 2**). In the same way, 1% bovine-derived material (meat- and bone-meal heated at 120 °C for 30 min) in concentrate feed mixtures was detected. This limit detection is higher than that of other methods such as Tartaglia et al. (*14*).

To identify beef in a large number of both processed and unprocessed foods, we carried out specific beef PCR amplifica-

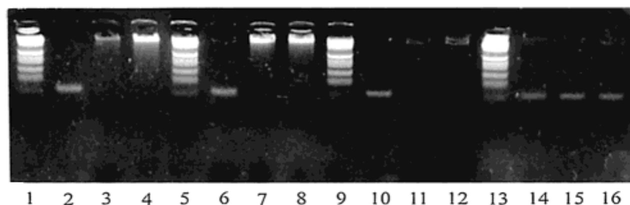

Figure 2. Sensitivity of the technique in different mixtures. Lines 1, 5, 9, and 13, BRL 1Kb marker (Gibco); lines 2–4, 1%, 0.1%, and 0.01% cattle feed mixtures, respectively; lines 6–8, 1%, 0.1%, and 0.01% beef in pork mixtures heated at 120 °C for 30 min; lines 10–12, 1%, 0.1%, and 0.01% beef in pork mixtures heated at 80 °C for 30 min; lines 14–16, 1%, 0.1%, and 0.01% raw beef in pork mixtures.

Table 3. Sensitivity of the Method in Cooked and Uncooked Pork and Beef Mixtures and Bovine-Derived Material in Feed by Specific Beef DNA Amplification

	100% → 1%	0.1%	0.01%	0.005%
pork and beef mixtures				
raw	+[a]	+	+	+
80 °C/30 min	+	−	−	−
120 °C/30 min	+	−	−	−
bovine-derived material in concentrate feed mixtures	+	−	−	−

[a] Beef detection by 84 bp amplification.

tion (**Table 2**). With this fragment, beef has been detected in mixtures treated at different temperatures, including autoclaved products (canned products). Some commercial products were analyzed which normally have beef or pork in their composition: chopped sausages, ham, salted ham, cured products. Using the specific PCR amplification, we detected beef in some sausages. Beef was not detected (**Table 2**) in hamburgers labeled as chicken, pork, and turkey.

In conclusion, specific PCR amplification of this fragment is a powerful technique for the identification of bovine contamination, due to its simplicity, specificity, and sensitivity (with 35 amplification cycles we can detect 0.005% raw beef).

However, further research would be needed in order to develop a quantitative method since this simple PCR analysis is only qualitative. With respect to beef quantification, the cause of a positive result should be clarified according to whether it is due to adulteration of the product or inadequate handling during manufacture in exceptional cases. In this way, Meyer et al. (*10*) do not consider it desirable to have a detection limit below 0.1%.

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