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Short communication. PCR detection of DNA of bovine, ovine-caprine and porcine origin in feed as part of a bovine spongiform encephalopathy control program

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Abstract

The rapid identification of residues of mammalian materials in animal feedstuffs is important for the effective control of feed as a potential source of transmission of bovine spongiform encephalopathy (BSE). It is included in the monitoring program developed to avoid the appearance of this disease. In the present work a PCR analysis was tested for the detection of mammalian residues (bovine, caprine, ovine and porcine). The DNA extraction method utilised guanidium thiocianate, and primers flanking a conserved region of mitochondrial DNA were synthesized for each species. PCR tests were specific for each species and allowed detection in feedstuffs of levels as low as 1% of bovine DNA and 1.5% of ovine, caprine and porcine DNA. These PCR tests may allow the rapid detection of residues of these species constituting a powerful tool against BSE.

Additional key words: bone meal, BSE, industrial samples, polymerase chain reaction.

Resumen

Comunicación corta. Detección mediante PCR de ADNs de origen bovino, ovino-caprino y porcino en concentrados y harinas, como parte del programa de control de la encefalopatía espongiforme bovina

La rápida identificación de restos de mamíferos en concentrados y harinas destinados a la alimentación animal es esencial para el control efectivo de esta fuente potencial de transmisión de la encefalopatía espongiforme bovina (EEB), como parte del programa de vigilancia que se desarrolla para evitar la aparición de esta enfermedad. En el presente trabajo se pusieron a punto ensayos de PCR para la detección de restos de mamíferos (bovino, caprino, ovino y porcino). El ADN se purificó utilizando tiocianato de guanidinio en el método de extracción y se sintetizaron cebadores específicos para cada especie, que amplifican una región altamente conservada del ADN mitocondrial (ADNm). Se determinó la sensibilidad y especificidad analítica en cada caso, resultando unos ensayos de PCR específicos para cada especie en particular, que permiten la detección en estos concentrados de hasta un 1% de material derivado de restos de bovino y un 1,5% de restos de ovino, caprino y porcino. Estos ensayos de PCR, que permiten la detección rápida de restos de estas especies de mamíferos, constituyen una poderosa herramienta en la lucha contra esta enfermedad.

Palabras claves adicionales: EEB, harina de hueso, muestras industriales, reacción en cadena de la polimerasa.

Bovine spongiform encephalopathy (BSE), also known as mad cow disease, has been detected in 26 countries including Canada and the United States. Consumption of meat from BSE-infected cattle is believed to have caused the death of nearly 200 people worldwide, from a disease called variant Creutzfeldt-Jakob disease (vCJD) (GAO, 2005).

Traditionally, animal species identification has been applied mainly for detection of commercial fraud, which involves substitution of an animal species of high commercial value, such as beef, by other species of lower commercial value. It is also a valuable tool for the assessment of risk associated with introduction of animal material that might be harmful to human or animal health. In this context it is widely accepted that BSE has spread through the consumption of contaminated animal feeds by healthy bovines (Wilesmith

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et al., 1998). For reasons of public health and disease prevention, the inclusion of meat and bone-meal (MBM) in ruminant feeds was banned in the United Kingdom in 1988 (Cheng *et al*., 2003). Similar regulations have been implemented in the EU and the USA (Baron *et al*., 1999). Recently, heat-stable proteins have been reported to be useful targets for both the detection of animal remains and species identification in foods of animal origin, such as meat (Chen and Hsieh, 2000) and fish products (Piñeiro *et al*., 2003). Methods based on DNA amplification are preferred, as they are less affected by industrial processing (Pascoal *et al*., 2005).

Generally, mitochondrial DNA (mtDNA) based PCR methods have given good results in analysis of samples submitted to temperature and pressure treatments, in which DNA has been partly degraded (Tartaglia *et al*., 1998; Wang *et al*., 2000; Bellagamba *et al*., 2001; Myers *et al*., 2001; Bottero *et al*., 2003; Rodríguez *et al*., 2004). PCR primers designed on the basis of sequences of short interspersed repetitive elements have been successfully used to detect low contamination levels of feed by animal material (Tajima *et al*., 2002). Recently, with the emergence of real-time PCR technology, PCR methods for mitochondrial encoded targets (Lahiff *et al*., 2002) or those in nuclear sequences (Brodmann and Moor, 2003) based on the use of Taqman probes, have been reported for the detection of bovine material in MBM and feedstuffs containing animal MBM (Castelló *et al*., 2004).

The aim of this work was to develop a simple, sensitive and accurate test, based on species-specific mtDNA amplification, to detect the presence of bovine, porcine and ovine/caprine contamination in feedstuffs. The study also aimed to establish the detection limit (sensitivity) and specificity of the PCR methods developed. Application of these species-specific PCR assays for detection of contaminating material from these species in industrial feedstuff samples was evaluated.

DNA was extracted as described by Boom *et al*. (1990) from 500 mg samples of i) bone meal, ii) feeds contaminated with different concentrations of bone meals, iii) industrial samples, and iv) negative controls.

The method is based on the DNA-binding properties of silica particles in the presence of guanidium thiocyanate (GuSCN), the latter being preferred to other chaotropic agents because of its nuclease-inactivating action. All reagents used were of molecular biology grade. To prevent DNA contamination, extractions were carried out in a dedicated laboratory. PCR set up was performed in a biological cabinet using dedicated pipette with aerosol barrier tips to ensure the risk of PCR contamination was minimal. PCR preparations and post-PCR analyses were conducted in separated rooms. Negative controls, lacking DNA or animal DNA were included in each extraction set.

Long bones from young animals (femur from cattle, goat, pig and sheep; tibia from chicken and spine from fish) were used. Bones were obtained shortly after slaughter by conventional boning and manual stripping with a scalpel. All samples were kept at –20°C until they were processed into flour. Bones were crushed in a mortar, dried in a vacuum (Bioblock Scientific) at 70°C for 24 h and milled in a micromill MFC at 3,000 rpm using a 1 mm sieve.

Feed samples contaminated with bone meal were prepared by mixing concentrated feed with processed bone meal of bovine, porcine, ovine and caprine origin at 0.05%, 0.1%, 0.5%, 1%, 1.5% and 2% concentrations. A control sample was prepared with no mammalian bone meal.

PCR primers for amplification of bovine DNA were designed from sequences available in the Genbank database (Acc. No. J01394) (Table 1). Species-specific primers for the detection of ovine/caprine and porcine DNA were as described by Lahiff *et al*. (2001) (Table 1).

PCR amplifications were performed in a final volume of 25 µl containing a final concentration of 0.25 mM deoxyribonuclotide triphosphates, 1X reaction buffer, 2.5 mM $MgCl_2$, 15 pmoles of each primer, 2.5 units of *Taq* polymerase (Promega) and 2.5 µl of purified DNA by the Boom *et al.* (1990) method.

The conditions of bovine DNA amplification assay were: a previous denaturing step at 95°C for 2 min; 10 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1.5 min; 20 cycles of 90°C for 1 min, 58°C for 1min, 72°C for 1.5 min; a final extension step at 72°C for 10 min. PCR for detection of porcine and ovine/caprine DNA was performed at 95°C for 2 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min; a final extension step at 72°C for 10 min. A template free negative control was included in each PCR run and sample preparation negative controls were included where appropriate.

Aliquots of 10 µl were analyzed by gel electrophoresis on 2% (w/v) agarose gel run in 1X TBE buffer, containing 0.5 g ml^{-1} ethidium bromide at 100-120 V for 1-2 h. A 100 bp DNA ladder marker (Promega) was used as size reference.

To test specificity, DNA samples from several animal species (cattle, sheep, goat, pig, fish and chicken) that may be present in feeds were analysed for the different

Table 1. PCR oligonucleotide primers

Primer sequence	Amplicon length (bp)	Source
Bovine		
B1: 5´CATCATAGCAATTGCCATAGTCC3' B2: 5´GTACTAGTAGTATTAGAGCTAGAATTAG3'	165	Genbank Acc No. J01394
Porcine		
P1: 5´GCCTAAATCTCCCCTCAATGGTA3' P2: 5´ATGAAAGAGGCAAATAGATTTTCG3'	212	Lahiff *et al.* (2001)
Ovine/caprine		
O1: 5´TTAAAGACTGAGAGCATGATA3' O2: 5´ATGAAAGAGGCAAATAGATTTTCG3'	225	Lahiff *et al.* (2001)

primers. To test the sensitivity, feed mixtures were made containing 0%, 0.05%, 0.1%, 0.5%, 1%, 1.5% and 2% bovine, porcine, ovine and caprine bone flours. The PCR was carried out under the same conditions as above.

Industrial samples were analyzed. Forty-five samples were analyzed for bovine presence, and the contaminated percentage was calculated. From these samples only seven were analyzed for the presence of porcine, and ovine/caprine material.

Figure 1A shows the amplification of bovine mitochondrial DNA fragment of 165 bp from processed bone meals. Figures 1B and 1C show the amplification of the extracted DNAs using porcine, ovine and caprine primers. The amplified products were of the appropriate size (212 bp for porcine DNA and 225 bp for ovine/caprine DNA). No PCR products were obtained, from the negative controls, with any of the species-specific primer sets.

Each of the primer pairs amplified DNA extracted from the species for which they were designed and showed no cross-reactivity with DNA from any other species (Figs. 1A, 1B, 1C). Thus, the novel primers designed in this work annealed specifically to the specific target DNA sequences but not to DNA from the other animal species analysed. The ovine specific primers amplified a 225 bp region from caprine DNA as well (Fig. 1C, lanes 2-3).

The sensitivity of the method was 1% for bovine, which corresponds to 100 pg of DNA, and 1.5% for porcine and ovine/caprine bone meal (Fig. 2).

When the primers were evaluated for the identification of bovine or porcine, ovine/caprine materials, in industrial feedstuffs, subjected to heating under pressure, the results were successful and showed the presence of bovine and porcine material in the samples (data not shown). After analysis of 45 industrial samples, PCR detected the presence of bovine material in 6 samples (13.3% contamination). Analysis of 7 of these samples for the presence of porcine DNA detected 2 positive samples (4.4% contamination in the whole samples). The two positive samples for porcine material were negative for bovine material. A total of 17.7% of ruminant feeds were contaminated with MBM. There were, however, no samples in which ovine/caprine materials were detected.

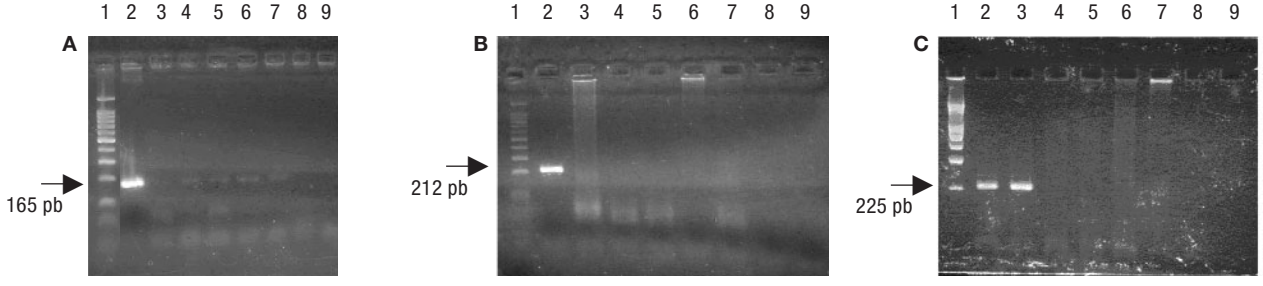

Figure 1. Specificity of the PCR assay for detection of bovine, ovine and caprine DNA using primers: A) B_1 and B_2, B) P_1 and P_2, C) O_1 and O_2. Lane 1: molecular weight marker 100 bp (A, B: Promega; C: Eurogentic); lane 2 (A: cattle, B: pig, C: goat); lane 3: (A: pig, B; cattle, C: sheep). 4: (A, B: goat, C: cattle); 5: A, B: sheep, C: pig); 6: chicken; 7: fish; 8: commercial wheat flour (negative control); 9: water control.

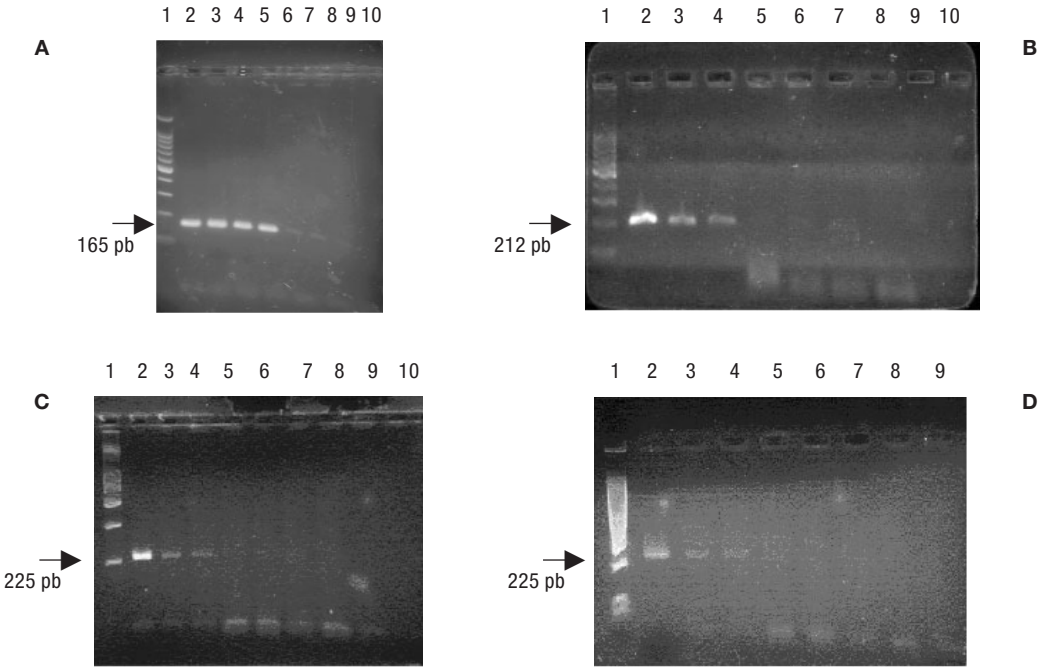

Figure 2. Sensitivity of the PCR assay using primers: A) B_1/B_2 for detection of bovine DNA, B) P_1/P_2 for detection of porcine DNA, C) O_1/O_2 for detection of ovine DNA, and D) O_1/O_2 for detection of caprine DNA. Lane 1: molecular weight marker 100 pb (Promega); 2: 100% (positive control); 3: 2%; 4: 1.5%; 5: 1%; 6: 0.5%; 7: 0.1%; 8: 0.05%; 9: commercial wheat flour (negative control); 10: water control.

Identification and/or differentiation of animal species has proved difficult, particularly in samples of complex composition which have been subjected to intensive processing (Laube *et al*., 2003). Although DNA exhibits fairly high thermal stability it is well known that intense heat coupled with high pressure conditions may cause severe DNA degradation, which affects the quality of the DNA recovered (Pascoal *et al*., 2005).

Analysis of mitochondrial DNA (mtDNA) has been reported to be a powerful tool for identifying and differentiating animal species in feeds (Cheng *et al*., 2003) because it has multiple copies per cell, then it increases the probability of achieving a positive result, even in samples with intense DNA fragmentation due to severe processing (Verkaar *et al*., 2002). Further, its high variability compared with nuclear sequences, which undergo a less rapid evolution, facilitates authenticity studies (Partis *et al*., 2000). Among mitochondrial targets, the *cytb* gene has frequently been considered a preferential DNA target for identification purposes (Prado *et al*., 2002). A PCR based method aimed at specific amplification of bovine specific mtDNA sequences from animal feeds has recently been tested and validated (Myers *et al*., 2001) as a way to ensure exclusion of bovine material from animal feeds.

Amplification of other PCR products has been developed for specific identification of bovine material by Momcilovic and Rasooly (2000) and Pascoal *et al*. (2005). The method developed here has potential because small size of the amplified fragment (165 bp) results in the successful DNA amplification in samples with intense DNA degradation caused by processing. Although previously reported bovine-specific primers have been based on other mitochondrial *cytb* sequences the PCR products in these studies had sizes of 274 bp (Matsunaga *et al*., 1999) or 285 bp (Herman, 2001). Rea *et al*. (2001) reported a bovine-specific PCR method aimed at amplification of a 113 bp *cytb* region, although this method was optimized for the identification of cow's milk. Pascoal *et al*. (2005) designed a PCR analysis that amplified a 115 bp *cytb* mitochondrial region. As in this method, small size is desirable to maximize the chance of getting positive results from samples which have been subjected to severe heat processing, as has suggested by Momcilovic and Rasooly (2000) and Pascoal *et al*. (2005).

The primers used for bovine detection are specific for this species and did not show any cross-reactivity (Fig. 1A). Lahiff *et al*. (2001) confirmed the specificity of porcine and ovine/caprine primers against a range

of DNA (bovine, ovine, porcine, horse, chicken, duck and *Salmo salar* DNA. In this work there was no cross reactivity with the DNA from the species investigated (Figs. 1B y 1C).

The sensitivity of the methods was 1% for bovine and 1.5% for porcine and ovine/caprine bone meal. Matsunga *et al.* (1999) found a detection limit of 0.25 ng of DNA with a multiplex PCR assay aimed at simultaneous PCR amplification of a region of the mitochondrial *cytb* gene from six species. Another study based on PCR amplification of the *cytb* gene gave a detection limit of 33.6 fg for DNA extracted from raw meat and had a 10 fold less sensitive detection limit for DNA extracted from cooked meat (Gouli *et al*., 1999).

Other studies have reported detection limits of 0.5% for bovine material (Seyboldt *et al*., 2003) or 1% (Sun and Lin, 2003), and for porcine and ovine material of 1% (Cheng *et al*., 2003), using a commercial kit for DNA purification. A range of different DNA extraction methods has been applied in different studies to extract PCR quality DNA from raw and cooked meat, meat and bone meat, animal feeds and canned foods.

The processing conditions to which commercial feedstuffs are subjected may involve either the presence of additives, which may inhibit DNA polymerase, or intense heating, which can degrade DNA to an extent that amplification is not possible (Bottero *et al*., 2003). As stated above, contamination with bovine material results in a significant risk for BSE spread.

Modern molecular techniques, based on DNA analysis, have good applicability in detecting adulteration, and they represent useful complements to methods relying on protein analysis for the identification animal species. DNA-based techniques have become effective and reliable for commercial dairy products also (Feligini *et al*., 2005).

Nucleic acid based analysis has been widely used in many fields, and has become increasingly popular for the differentiation and identification of feed or food adulterants (Tartaglia *et al*., 1998; Partis *et al*., 2000). The advantages of DNA-based analysis are manifold. First is the ubiquity of DNA: that from all cell type of an individual contains identical genetic information. Secondly, the information content of DNA is more abundant compared to proteins due to the degeneracy of the genetic codes. Thirdly, DNA is a rather stable molecule which renders DNA extraction and analysis from many sample types feasible (Cheng *et al*., 2003).

A detection method based on mtDNA can further improve sensitivity because each cell has only a single set of genomic DNA in the nucleus, but several copies of mtDNA. The mtDNA has a high mutation rate, because of its location in the cytoplasm and susceptibility to attack; there is poor corrective replication of polymerase and a lack of a proof-reading system in mitochondria. There is a specificity of mtDNA expressed in different species or genera and individual species can be identified by studying mtDNA. There are approximate 1,000 mitochondria in a cell and 10 copies of mtDNA per mitochondrion, thus, 10^4 copies of mtDNA are available per cell compared with just one copy for genomic DNA. Therefore, it is more efficient to detect species-specific DNA using mtDNA than genomic DNA (Cheng *et al*., 2003).

The PCR assays described here are suitable routine testing of industrial feeds. They are rapid, simple and applicable for detection of DNA from bovine, porcine, ovine and caprine origin, up to 1% and 1.5% contamination, respectively.

Such contamination may result from carryover ocurringin the feed mixer after mixing monogastric feeds, which are occasionally supplemented with MBM as a protein source. The results showed that the PCR methods described can provide a rapid, reproducible and sensitive procedure to detect MBM contamination in feeds.

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