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Identification of Species-Specific DNA in Feedstuffs

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Due to the menace of transmission of spongiform encephalopathies, feed components intended for ruminant nutrition must be checked for the presence of ruminant-derived materials. A sensitive method for the identification of bovine- and ovine- and also swine- and chicken-specific mitochondrial DNA sequences based on Polymerase Chain Reaction (PCR) has been developed. The specificity of the primers for PCR has been tested using samples of DNA of other vertebrate species, which may also be present in rendering plant products intended for feed manufacture. The method allows the detection in concentrate mixtures of 0.01% of the target species derived material. The identity of a sample containing 0.1% of bovine, ovine, swine, and chicken meat-and-bone meal has further been confirmed by sequencing.

KEYWORDS: Bovine spongiform encephalopathy; feed; mitochondrial DNA; Polymerase Chain Reaction

INTRODUCTION

European countries are still menaced by bovine spongiform encephalopathy (BSE). This transmissible disease was first diagnosed in Great Britain in 1986 (*1*) and subsequently in a number of European countries including the Czech Republic. Experimental studies have demonstrated that BSE is a variant of the sheep disease scrapie and that transmission to cattle resulted from feeding of cattle with processed slaughterhouse waste containing sheep tissues (*2*). The transmission via feed was confirmed experimentally by feeding domestic goats with brain tissue collected from BSE-positive cattle (*3*). Therefore, the EU issued a ban to feed ruminant tissues to ruminants. Authorities of the Czech Republic, where the first two separate cases of BSE were diagnosed in 2001, issued a ban to feed ruminants with meat-and-bone meals originating from cattle, sheep, swine, or chickens, in 1991. Currently, European Union (EU) legislation prohibits the inclusion of meat-and-bone meal in feeds for all animals used for human consumption (decision 2000/766/EC) (*4*).

Monitoring of the observation of this ban is difficult because of the lack of a routine method for the identification of components of meat-and-bone meals. The presence of meat-and-bone meal in feeds can be demonstrated by microscopic examination. Sedimentation analysis followed by microscopy can detect constituents of animal origin in feedstuffs at concentrations as low as 0.1%. However, it is generally impossible to identify the species involved (*5*). The application of conventional immunoenzymatic methods to species identification of meat-and-bone meals fails to detect heat-treated proteins (*6*, *7*). So far, only one laboratory has succeeded in the development of an immunoassay for the detection of ruminant and porcine proteins at 0.125% concentration in experimental meat-and-bone meals heated to 130 °C at 2.7 bar incorporated into compound animal feedstuffs (*8*).

Molecular biological techniques allow the demonstration of DNA in heat-processed samples. They can also be used for species-specific identification of DNA in meat-and-bone meal and in compound feeds. The first to describe such applications were Tartaglia et al. (*9*), who used Polymerase Chain Reaction (PCR) for the amplification of bovine mitochondrial DNA sequences as a marker for the presence of bovine-derived tissues in feeds. PCR for the demonstration of ovine, porcine, and poultry DNA in experimental meat-and-bone meal was also described, but the detection limit for the meal was 0.3−1% and no data on the confirmation of the PCR product were given (*10*). In another recent paper, a PCR−restriction fragment length polymorphism technique (RFLP) for species identification in rendered animal material with a sensitivity of 0.5% meat meal in feedstuffs has been presented (*11*). An interspersed repetitive element method for specific measurement of ruminant, pig, and chicken DNA in feed is newly published (*12*).

Our objective was to develop a sensitive and reliable PCR method for the routine identification of bovine, ovine, swine, and chicken DNA in meat-and-bone meal intended for feed manufacture.

MATERIALS AND METHODS

DNA Extraction. DNA was isolated from reference feed samples according to a method based on the binding of DNA with SiO_2 in guanidine thiocyanate (GuSCN) (*9*, *13*). Two hundred milligrams of the sample was transferred into a 2 mL test tube and mixed with 1 mL of extraction buffer (5 M GuSCN; 0.05 M Tris-HCl, pH 6.4; 0.02 M EDTA, pH 8.0; 1.3% Triton X-100), and glass beads (2.5 mm) were added to fill the test tube. The mixture was homogenized on a Mini Beadbeater (BioSpec Products, Bartlesville, OK) twice for 20 s with the speed at 4600 rpm. Thereafter, the mixture was centrifuged (14000*g*, 15 min); 200 μL of the supernatant was transferred into the 1.5 mL test tube and mixed with 500 μL of extraction buffer and 40 μL of

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Table 1. Oligonucleotide Tested To Amplify the Species-Specific Mitochondrial Fragment Encoding for the tRNA[Lys] and ATPase 8- and 6-Subunit Sequences

primer[a]		oligonucleotide	product length (bp)
bovine	F8108	5′-CCATATACTCTCCTTGGTGAC-3′	270
	R8357	5′-GTAGGCTTGGGAATAGTACGA-3′	
ovine	F7864	5′-ACACAACTTCTACCACAACCC-3′	145
	R7988	5′-AAACAATGAGGGTAACGAGGG-3′	
swine	F7773	5′-CTCAATGGTATGCCACAACTAG-3′	313
	R8064	5′-CATTGTTGGATCGAGATTGTGC-3′	
chicken	F9097	5′-AACCCAAACCCATGATTCTCC-3′	206
	R9282	5′-GAGGATTAGAGGGATTCCTAG-3′	

[a] The primers are identified by letters designating the forward (F) or reverse (R) strand and a number corresponding to the position of the base at the 5′ end of the primer in the bovine, ovine, swine, and chicken reference sequences, according to the methods of Anderson et al. (*14*), Hiendleder et al. (*15*), Ursing and Arnason (*16*), and Desjardins and Morais (*17*), respectively.

silica suspension (prepared on the preceding day by mixing 100 mg of Celite with 500 μL of water and 5 μL of 32% hydrochloric acid). The mixture was incubated at room temperature for 10 min and centrifuged (14000*g*, 1 min) and the supernatant was decanted. The silica pellet was washed once with 500 μL of washing buffer (5 M GuSCN; 0.05 M Tris-HCl, pH 6.4), once with 500 μL of 70% ethanol, and once with 500 μL of acetone. The content of the uncovered test tube was dried in an incubator at 56 °C for 15 min, mixed with 70 μL of tempered (56 °C) water, incubated at room temperature for 2 min, and centrifuged (14000*g*, 1 min); 40 μL of the supernatant was transferred into another test tube, centrifuged again (14000*g*, 1 min), and used in PCR.

DNA samples of domestic cattle, domestic sheep, domestic goat, domestic swine, domestic horse, domestic rabbit, domestic chicken, domestic turkey, domestic duck, carp, and rainbow trout were isolated according to the procedure FastPrep Instrument (Thermo Savant, Carlsbad, CA). The homogenization was performed with one $^1/_4$ in. ceramic sphere, speed 5.0 for 30 s, and DNA was isolated in 0.5 mL of extraction buffer as described above. Sample sizes were 50 mg of muscular tissues. The fish meal was operated in the same way as the negative control.

PCR. For the detection of bovine species, the primers described by Tartaglia et al. (*9*) were used. The other primer pairs for ovine, swine, and chicken in the regions of mitochondrial tRNA[Lys] and ATPase subunits 8 and 6 were designed (**Table 1**), and the following combinations were used: F8108/R8357 and F7864/R7988 for bovine and ovine DNA duplex amplification, and F7773/R8064 and F9097/R9282 for swine and chicken DNA duplex amplification, respectively.

The amplifications of the feed samples were carried out in a final volume of 20 μL in a reaction mixture containing 2 μL of solution of primers, 10 μL of HotStarTaq Master Mix (Qiagen GmbH, Hilden, Germany), 1.2 μL of 25 mM $MgCl_2$, 4.8 μL of water, and 2 μL of sample. The final concentration of the solution of each set of primers was 100 nM for ovine and 400 nM for bovine duplex amplification and 100 nM for chicken and 400 nM for pig duplex amplification, respectively. The amplifications were run in the PTC-200 DNA engine cycler (MJ Research Inc., Watertown, MA) using the following program: initial denaturation step at 95 °C for 10 min followed by 35 cycles at 94 °C for 15 s, 58 °C for 15 s, and 72 °C for 15 s, and closed by the extension step at 72 °C for 3 min. The amplifications of DNA samples from the muscular tissues of the individual animal species were carried out in the same way except for reduction of the number of cycles to 25. The amplified DNA was determined by electrophoresis in 2% agarose gel and staining with Sybr Gold (Molecular Probes Inc., Eugene, OR).

Dilution Series. Series of model grain concentrates containing various amounts of bovine, ovine, swine, and chicken meat-and-bone meal (all processed at 133 °C, 300 kPa, 30 min) were prepared as follows: 4 g of grain concentrate was mixed with 250 mg of 100% bovine, ovine, swine, and chicken meat-and-bone meal, and the mixture was homogenized in a mortar; 1 g of the homogenate was mixed with

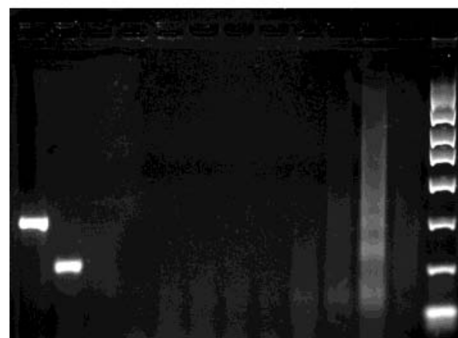

Figure 1. Amplification of DNA of various animal species with primers for bovine and ovine DNA: (lane 1) domestic cattle; (lane 2) domestic sheep; (lane 3) domestic goat; (lane 4) domestic swine; (lane 5) domestic horse; (lane 6) domestic rabbit; (lane 7) domestic chicken; (lane 8) domestic turkey; (lane 9) domestic duck; (lane 10) carp; (lane 11) rainbow trout; (lane 12) fish meal; (lane 13) molecular weight marker (Sigma Chemical Co., St. Louis, MO), fragments 2000, 1500, 1000, 750, 500, 300, 150, and 50 base pairs.

4 g of grain mixture and homogenized. In the next step 500 mg of the homogenate was mixed with 4.5 g of grain mixture and homogenized, and this procedure was repeated to obtain a dilution series of grain concentrates containing 5, 1, 0.1, and 0.01% of bovine, ovine, swine, and chicken meat-and-bone meal. Also, the mixture containing 5% of ovine and chicken and 0.009% of bovine and swine meat-and-bone meal by mixing of 902 mg of 0.01% above grain concentrate with 49 mg of ovine and chicken meat-and-bone meal was prepared. In the same way the mixture containing 5% of bovine and swine and 0.009% of ovine and chicken meat-and-bone meal was prepared.

PCR Product Sequencing. The reaction mixture (50 μL) contained always one set of primers (final concentration was 200 nM) for each species, 3.0 μL of 25 mM $MgCl_2$, 25 μL of HotStarTaq Master Mix, and 6 μL of sample 0.1%. Four different reactions, each with one set of primers, were performed. The amplification was run using the same program as in the preceding PCR for feed samples. The obtained product was cleaned on the Centri Spin 10 column (Princeton Separation Inc., Adelphia, NJ) and used without any concentration adjustment in the sequenation mixture (20 μL), which contained always 4 μL of 0.85 μM solution of forward primer for each species, 8 μL of BigDye Terminator Ready Reaction Mix (Applied Biosystems, Warrington, U.K.), and 8 μL of purified PCR product. The amplification was run using the following program: 25 cycles at 96 °C for 15 s, 50 °C for 15 s, and 60 °C for 4 min. The product was cleaned on the Centri Sep column and processed in the Abi Prism 310 genetic analyzer.

RESULTS AND DISCUSSION

The objective of our study was to develop a rapid, sensitive, and reliable PCR procedure for the demonstration of bovine, ovine, swine, and chicken meat-and-bone meal in compound feeds.

DNA was isolated using the method based on binding of DNA with SiO_2 in the presence of guanidin thiocyanate (*9*, *13*). This method has been found to be effective for the isolation of DNA from such materials because it removes inhibitors of PCR and its yield is sufficient. Sample homogenization with the help of the glass beads is very quick, and the release of DNA requires only several seconds, in contrast to the overnight water bath incubation (*18*).

Three primer pairs were designed for the region of ovine, swine, and chicken mitochondrial tRNA[Lys] and ATPase subunits 8 and 6, and the size of the amplification products ranged from 145 to 313 bp. The choice of the primers was based on results of a method for the determination of bovine-specific DNA (*9*).

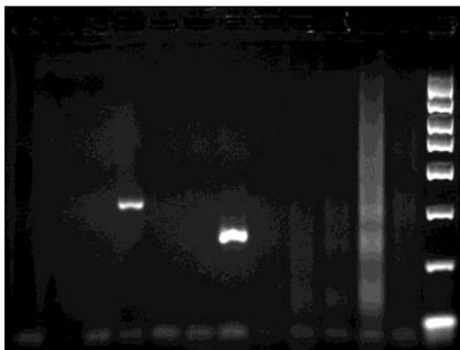

Figure 2. Amplification of DNA of various animal species with primers for swine and chicken DNA: (lane 1) domestic cattle; (lane 2) domestic sheep; (lane 3) domestic goat; (lane 4) domestic swine; (lane 5) domestic horse; (lane 6) domestic rabbit; (lane 7) domestic chicken; (lane 8) domestic turkey; (lane 9) domestic duck; (lane 10) carp; (lane 11) rainbow trout; (lane 12) fish meal; (lane 13) molecular weight marker, fragments 2000, 1500, 1000, 750, 500, 300, 150, and 50 base pairs.

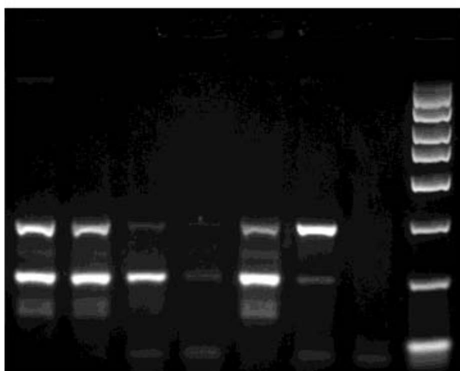

Figure 3. Dilution series: amplification of primers for bovine and ovine DNA: (lanes 1–4) grain concentrates containing 5, 1, 0.1, and 0.01% of bovine, ovine, swine, and chicken meat-and-bone meal, respectively; (lane 5) grain concentrates containing 5% of ovine and chicken meat-and-bone meal and 0.009% of bovine and swine meat-and-bone meal; (lane 6) grain concentrates containing 5% of bovine and swine meat-and-bone meal and 0.009% of ovine and chicken meat-and-bone meal; (lane 7) feed alone; (lane 8) molecular weight marker, fragments 2000, 1500, 1000, 750, 500, 300, 150, and 50 base pairs.

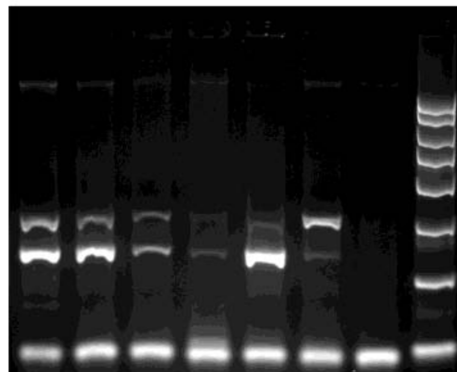

Figure 4. Dilution series: amplification of primers for swine and chicken DNA: (lanes 1–4) grain concentrates containing 5, 1, 0.1, and 0.01% of bovine, ovine, swine, and chicken meat-and-bone meal, respectively; (lane 5) grain concentrates containing 5% of ovine and chicken meat-and-bone meal and 0.009% of bovine and swine meat-and-bone meal; (lane 6) grain concentrates containing 5% of bovine and swine meat-and-bone meal and 0.009% of ovine and chicken meat-and-bone meal; (lane 7) feed alone; (lane 8) molecular weight marker, fragments 2000, 1500, 1000, 750, 500, 300, 150, and 50 base pairs.

For the determination of species-specific DNA, the authors recommend the region of mitochondrial DNA containing genes for $tRNA^{Lys}$ and ATPase subunits 8 and 6 containing sequences specific for individual mammalian species (*9*, *19*, *20*). The primers were also designed for duplex amplification of bovine and ovine DNA and swine and chicken DNA, respectively. The specificity of the designed primers was tested using samples of DNA of 10 animal species that may be present in meat-and-bone meals. Fish meal was used as a negative control in the isolation and amplification experiments. All of the suggested primer pairs yielded specific amplification products (**Figures 1** and **2**).

A dilution series was created using samples containing known concentrations of bovine, ovine, swine, and chicken meat-and-bone meal within the range from 5 to 0.01%. The possibility of duplex PCR was verified in case the concentration of the determined meat-and-bone meals was 5 or 0.009% (**Figures 3** and **4**). Our detection limit of 0.01% is identical with that reported by Tajima et al. (*12*), who applied the interspersed repetitive DNA element for the detection of ruminant, pig, and chicken meat-and-bone meal. These authors have also emphasized the importance of the sensitivity of the method for the demonstration of cross-contamination in field samples.

The identity of the sample containing 0.1% of bovine, ovine, swine, and chicken meat-and-bone meal was confirmed by direct sequencing of the PCR product.

It can be concluded that the method described in this paper is suitable for testing of compound feeds and their components for the presence of bovine, ovine, swine, and chicken DNA.

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