Journal of Food Protection, Vol. 68, No. 6, 2005, Pages 1217–1221

Quantitative Detection of Species-Specific DNA in Feedstuffs and Fish Meals

PAVEL KRCMAR* AND EVA RENCOVA

Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic

MS 04-458: Received 29 September 2004/Accepted 5 February 2005

ABSTRACT

A sensitive and rapid method for the quantitative detection of bovine-, ovine-, swine-, and chicken-specific mitochondrial DNA sequences based on real-time PCR has been developed. The specificity of the primers and probes for real-time PCR has been tested using DNA samples of other vertebrate species that may also be present in rendered products. The quantitative detection was performed with dual-labeled probes (TaqMan) using absolute quantification with external standards of single species meat-and-bone meals. This method facilitates the detection of 0.01% of the target species–derived material in concentrate feed mixtures and fish meals.

Bovine spongiform encephalopathy was first diagnosed in Great Britain in 1986 *(24).* It still occurs in many European countries including the Czech Republic, where it was confirmed in 2001 for the first time, with 17 cases having been detected so far. Authorities of the Czech Republic issued a ban on feed products containing bone meals and meat-and-bone meals originating from ruminants in 1991. The European Union (EU) issued the first ban on feed protein derived from ruminant tissues that was targeted to be fed to ruminant species in 1994 *(2).* The current EU legislation prohibits feeding protein derived from mammals to any farm animal species *(4).*

Measuring compliance with this ban is difficult because of the lack of suitable methods for the identification of animal protein, which may enter the feed chain mainly in the form of a meat-and-bone meal. The only EU official method available for the detection of meat-and-bone meal in animal feeds is microscopic examination *(3).* The method is based on the discrimination between mammalian, poultry, and fish bones. The detection limit of this method is about 0.1% of meat-and-bone meal when present in feeds. However, the method depends on the experience of the analyst and is time-consuming and laborious *(14).* Immunoassays for the detection of ruminant and porcine proteins at 0.125% concentration and bovine and ovine proteins with a detection limit of 5.0 and 4.0 ng/ml, respectively, in experimental meat-and-bone meals incorporated into feedstuffs have been developed *(5, 10).* Today, immunoassays are used primarily as screening methods, but they are not sufficiently specific to be used as confirmation tests for forensic identification *(14).*

Molecular genetic methods are capable of detection of species-specific DNA in heat-processed samples. The first studies that described such tests for the identification of meat-and-bone meal in feedstuffs were those by Tartaglia et al. *(22),* who used PCR for the amplification of bovine mitochondrial DNA (mtDNA) sequences. The method was validated by four laboratories in the United States *(19).* Frezza et al. *(13)* investigated the relationship between bovine mtDNA degradation, PCR amplification, and heat treatment of meat-and-bone meal. Colgan et al. *(11)* described the use of PCR for the demonstration of ovine, porcine, and poultry mtDNA in experimental meat-and-bone meal, with the detection limit being 0.3 to 1% of the target species when present in feed products. A study that describes the use of PCR to detect long and short interspersed repetitive elements of ruminant, pig, and chicken DNA in feed has been published *(21).* Myers et al. *(20)* have suggested a PCR primer set that is capable of amplifying mtDNA segments from various species (cattle, sheep, goat, deer, elk, pig, and horse) for the simultaneous detection of animal-derived material in feed. Bellagamba et al. *(6)* presented a PCR–restriction fragment length polymorphism (RFLP) technique using universal primers for the cytochrome *b* gene of mtDNA for species identification in rendered animal material with a sensitivity of 0.5% of the target species when present in feedstuffs. Bellagamba et al. *(7)* also developed a PCR method based on the 12S ribosomal RNA gene of mtDNA, which specifically detects 0.125% beef, sheep, pig, and chicken and 0.5% goat tissue when present in laboratory-prepared in fish meals. Recently, real-time PCR techniques for the detection of bovine DNA in meat-and-bone meal samples have been reported. Lahiff et al. *(18)* used the primers published by Tartaglia et al. *(22)* and designed a dual-labeled probe. Brodmann and Moor *(9)* tested primers and dual-labeled probes that can be used for the detection of the beef and mammal growth hormone gene.

Because of the heat degradation of DNA under meat-and-bone meal processing, it is necessary to use a shorter amplicon size for PCR amplification. So the use of a real-time PCR method, which requires an amplicon size no larg-

* Author for correspondence. Tel: 4205 3333 1815; Fax: 4205 4121 1229; E-mail: krcmar@vri.cz.

TABLE 1. *Oligonucleotide tested to amplify the species-specific mitochondrial fragment encoding for the tRNA^{Lys} and ATPase 8- and 6-subunit sequences*

	Primer and probe[a]	Oligonucleotide	Product length (bp)
Bovine	F8108	5′-CCATATACTCTCCTTGGTGAC-3′	144
	R8231	5′-TGGTGTCAGTTCTGGATTGTG-3′	
	P8135	5′-CAACTAGACACGTCAACATGACTG-3′	
Ovine	F7864	5′-ACACAACTTCTACCACAACCC-3′	145
	R7988	5′-AAACAATGAGGGTAACGAGGG-3′	
	P7904	5′-ACACCGAAACAAAATACTCCTTGAGAAACA-3′	
Swine	F7975	5′-CCCCACGATAATAGGACTACCT-3′	111
	R8064	5′-CATTGTTGGATCGAGATTGTGC-3′	
	P8021	5′-CCAAGCTTACTATTCCCAACACCCAAACGA-3′	
Chicken	F9188	5′-ACAACCCTGCAAACAAAATTACAA-3′	113
	R9277	5′-GGATTAGAGGGATTCCTAGTAGGC-3′	
	P9231	5′-CCCCTGAACCTGACCATGAACCTAAGCT-3′	

[a] The primers and probes are identified by letters designating the forward strand (F), the reverse strand (R), or the probe (P) and a number corresponding to the position of the base at the 5′ end of the positive strand of the primer or probe in the bovine, ovine, swine, and chicken reference sequences, according to Anderson et al. *(1)*, Hiendleder et al. *(15)*, Ursing and Arnason *(23)*, and Desjardins and Morais *(12)*, respectively.

er than 150 bp, is very useful. This method also enables the utilization of species-specific probes, thus increasing the specificity of the amplification. In this case, it is usually not necessary to perform the measurement of the PCR product by gel electrophoresis or to confirm its composition using RFLP techniques or sequencing; thus, the analysis time can be shortened significantly. The continual measurement of fluorescence corresponds to the amount of product in real-time PCR and also enables its quantitative determination. We have decided to exploit these advantages of real-time PCR for the development of a method for the determination of meat-and-bone meal prepared at rendering plant under EU conditions.

The goal of our study was to devise a sensitive, rapid, and reliable PCR test for the routine quantitative detection of bovine, ovine, swine, and chicken meat-and-bone meals in feeds and fish meals.

MATERIALS AND METHODS

DNA isolation. DNA was isolated from reference feed and fish meal samples by a method based on the binding of DNA with diatom in guanidine thiocyanate (GuSCN) *(8, 22)*. A total of 200 mg of the sample of feed or 100 mg of fish meal was transferred to a 2-ml test tube and mixed with 1 ml of lysis 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 2.5-mm glass beads were added to each test tube. The mixture was homogenized in a Mini Beadbeater (BioSpec Product, Bartlesville, Okla.) twice for 20 s at 4,600 rpm. Then, the mixture was centrifuged (14,000 × *g*, 15 min), and 200 μl of the supernatant was transferred into a 1.5-ml test tube, mixed with 500 μl of lysis buffer and 40 μl of a diatom suspension (prepared on the preceding day by mixing 100 mg of Celite with 500 μl of water and 5 μl of 32% HCl), and vortexed for 15 s. The mixture was incubated at room temperature for 10 min and centrifuged (14,000 × *g*, 1 min), and then the supernatant was decanted. The diatom 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 (14,000 × *g*, 1 min); next, 40 μl of the supernatant was transferred to a 0.5-ml test tube, centrifuged again (14,000 × *g*, 1 min), and used in real-time PCR.

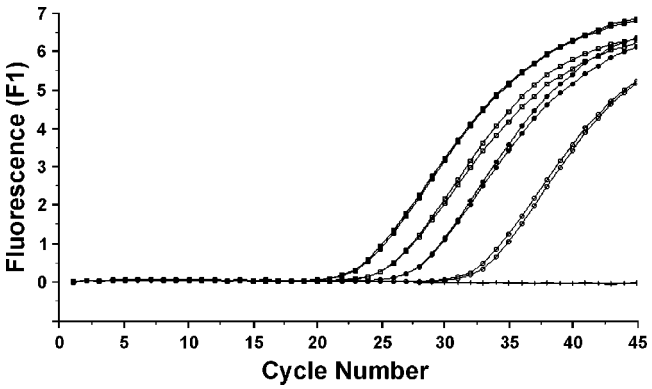

FIGURE 1. *Calibration curve for amplification of bovine DNA in grain concentrates containing 5 (■), 1 (□), 0.1 (●), and 0.01% (○) of bovine, ovine, swine, and chicken meat-and-bone meal, respectively; present in feed alone (|).*

DNA samples of cattle, sheep, goat, swine, horse, rabbit, chicken, turkey, duck, and goose, as well as carp and rainbow trout, were isolated from 50 mg of the muscular tissues. The samples were put into a 1.5-ml test tube, mixed with 0.5 ml of lysis buffer, vortexed for 15 s, and incubated in a water bath at 60°C for 17 to 21 h. The mixture was centrifuged (14,000 × *g*, 15 min), and 200 μl of the supernatant was transferred into a 1.5-ml test tube and mixed with 500 μl of lysis buffer and 40 μl of diatom suspension; DNA was then isolated as described above.

Real-time PCR. For the detection of species-specific DNA, the primers and probes in the regions of mitochondrial tRNA^{Lys} and ATPase subunits 8 and 6 were designed (Table 1), and the following combinations were used: F8108, R8231, and P8135 for bovine DNA amplification; F7864, R7988, and P7904 for ovine DNA amplification; F7975, R8064, and P8021 for swine DNA amplification; and F9188, R9277, and P9231 for chicken DNA amplification. The amplifications of the feed and fish meal samples were carried out in a final volume of 20 μl in a reaction

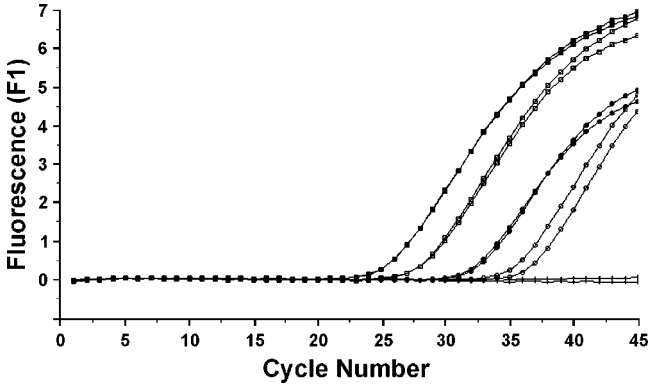

FIGURE 2. *Calibration curve for amplification of bovine DNA in fish meal containing 5 (■), 1 (□), 0.1 (●), and 0.01% (○) of bovine, ovine, swine, and chicken meat-and-bone meal, respectively; present in fish meal alone (|).*

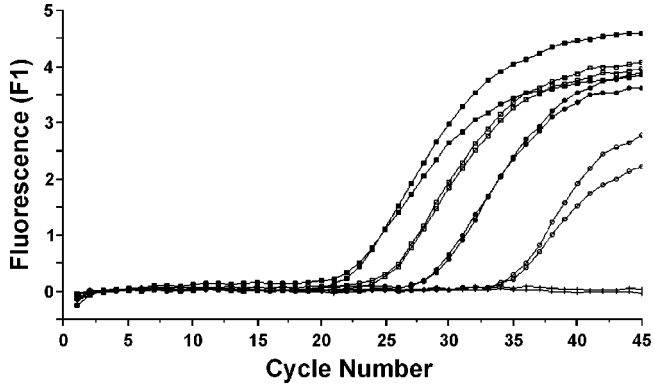

FIGURE 4. *Calibration curve for amplification of ovine DNA in fish meal containing 5 (■), 1 (□), 0.1 (●), and 0.01% (○) of bovine, ovine, swine, and chicken meat-and-bone meal, respectively; present in fish meal alone (|).*

mixture containing 10 μl of QuantiTect Probe PCR Master Mix (Qiagen GmbH, Hilden, Germany), a 2-μl solution of primers and probe, 6 μl of water, and 2 μl of sample. The final concentration of the solutions of each primer and the probe was 500 and 100 nM, respectively. All probes were labeled with a 5′ FAM (carboxyfluorescein) reporter dye and a 3′ Black Hole 1 quencher. The amplifications were run in a LightCycler amplifier (Roche Diagnostics GmbH, Mannheim, Germany) using the following program: uracil-*N*-glycosylase treatment at 50°C for 2 min and the initial activation step at 95°C for 15 min, followed by 45 cycles at 95°C for 0 s and at 60°C for 60 s. All amplifications of the feed and fish meal samples were performed in duplicate. The amplifications of DNA samples from the muscular tissues of individual animal species were carried out in the same way, except for a reduction in the number of cycles to 35.

Calibration curve. A series of model grain concentrates containing various amounts of bovine, ovine, swine, and chicken meat-and-bone meals were prepared as follows: 4 g of grain concentrate was mixed with 250 mg of bovine, ovine, swine, and chicken meat-and-bone meal, and the mixture was homogenized in a mortar; 1 g of the homogenate mixture was mixed again with 4 g of grain concentrate and homogenized. In the next step, 500 mg of the homogenate mixture was mixed with 4.5 g of grain concentrate 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. The series of fish meal samples containing 5, 1, 0.1, and 0.01% of bovine, ovine, swine, and chicken meat-and-bone meal were prepared in the same way. Every sample contained equal amounts of bovine, ovine, swine, and chicken meat-and-bone meal.

All meat-and-bone meals were processed at 133°C, 300 kPa, for 30 min (EU conditions). Bovine, swine, and chicken meat-and-bone meals were obtained from a rendering plant (Zichlinek Company, Czech Republic), with each species being specially prepared separately under our request. Ovine (lamb) meat-and-bone meal was obtained from Taranaki By Products Ltd. (Kohiti Road, Okaiawa, New Zealand). Bovine concentrates consisted of cereals and oil plants and their by-products, minerals, and vitamins.

RESULTS AND DISCUSSION

The purpose of the present study was to develop a sensitive, rapid, and reliable PCR method for the routine quantitative detection of bovine, ovine, swine, and chicken meat-and-bone meal in feeds and fish meals. On the basis of our previous experiments regarding the positive identification of bovine meat-and-bone meal in fish meals (unpublished data), a method for determining the possible adulteration of fish meals was also developed.

DNA was isolated using the method that is based on

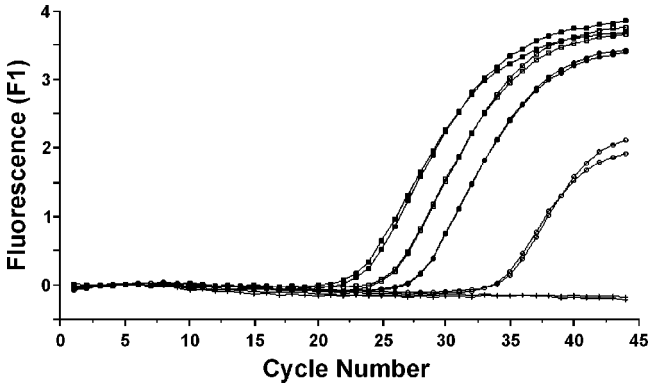

FIGURE 3. *Calibration curve for amplification of ovine DNA in grain concentrates containing 5 (■), 1 (□), 0.1 (●), and 0.01% (○) of bovine, ovine, swine, and chicken meat-and-bone meal, respectively; present in feed alone (|).*

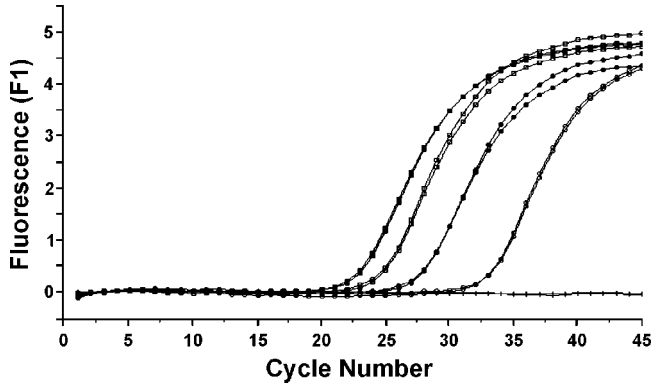

FIGURE 5. *Calibration curve for amplification of swine DNA in grain concentrates containing 5 (■), 1 (□), 0.1 (●), and 0.01% (○) of bovine, ovine, swine, and chicken meat-and-bone meal, respectively; present in feed alone (|).*

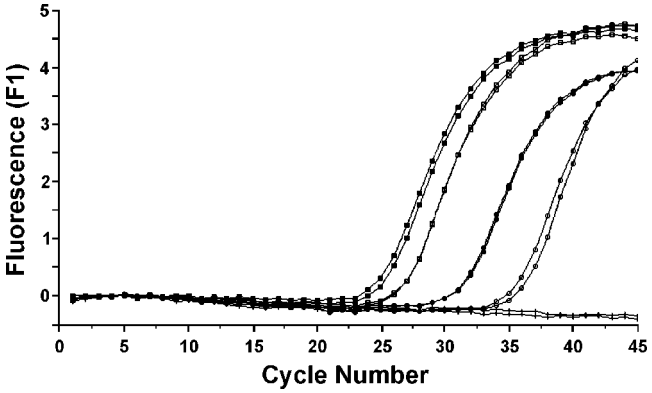

FIGURE 6. *Calibration curve for amplification of swine DNA in fish meal containing 5 (■), 1 (□), 0.1 (●), and 0.01% (○) of bovine, ovine, swine, and chicken meat-and-bone meal, respectively; present in fish meal alone (|).*

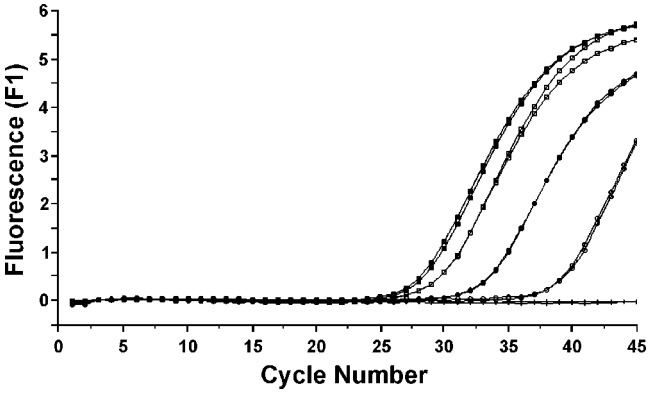

FIGURE 7. *Calibration curve for amplification of chicken DNA in grain concentrates containing 5 (■), 1 (□), 0.1 (●), and 0.01% (○) of bovine, ovine, swine, and chicken meat-and-bone meal, respectively; present in feed alone (|).*

the binding of DNA with diatomic material in the presence of GuSCN. This method has already proven useful in our previous experiments for the isolation of animal DNA from meat-and-bone meals, as it facilitates the ability to obtain a sufficient amount of DNA while removing PCR inhibitors. Sample homogenization by means of glass beads is a very fast process; thus, the time of sample lysis is shortened to a few seconds in comparison with an overnight water bath incubation *(16, 17).* The amount of fish meal sample used for analysis was only 100 mg; when higher amounts were used, high-viscosity solutions were obtained. Some of the steps required under the DNA isolation procedure made pipetting 200 μl the resulting viscous supernatant impossible.

For the detection of bovine DNA, the forward primer that has been described by Tartaglia et al. *(22)* was used. Since a product length of less than 150 bp is preferred in real-time PCR, a reverse primer was designed that would amplify a shorter DNA sequence than the original 271-bp PCR product. Three primer pairs were designed for the regions of ovine, swine, and chicken mitochondrial ATPase subunits 8 and 6, and the size of the amplification products from these primers ranged from 111 to 145 bp (Table 1). The choice of primers was based on a method for the determination of bovine-specific DNA *(22)* and our previous experiments *(17).* For the determination of species-specific DNA, the authors recommend using a region of mtDNA that contains genes for $tRNA^{Lys}$ as well as genes for ATPase subunits 8 and 6 that contain sequences specific for the particular species of animal being investigated. Dual-labeled probes were designed for increasing specificity of amplification as well as for quantitative detection of the amount of amplified DNA. The specificity of the designed primers and probes was tested using samples of DNA from 11 animal species that may be present in meat-and-bone meals. All the designed primer pairs and probes yielded specific amplification products (data not shown).

Quantitative determination facilitates better monitoring of feed and fish meal. As the amount of adulteration in the feed and fish meal used to feed ruminants increases, so does the risk of potential infections. For the preparation of calibration curves, we used single-species meat-and-bone meals manufactured in rendering plants. Calibration curves were prepared using feed and fish meal samples containing known concentrations of bovine, ovine, swine, and chicken meat-and-bone meals that ranged from 5 to 0.01% (Figs. 1 through 8). The regression coefficient (r) ranged from −0.95 to −1.00 in all experiments. The detection limit was 0.01% of bovine, ovine, swine, and chicken meat-and-bone meal when present in feed or fish meal. An identical detection limit was obtained both in our previous study *(17)* and in the study by Tajima et al. *(21),* who reported the identification of ruminant, swine, and chicken meat-and-bone meal when present at a concentration of 0.01% in feed. In other laboratory-scaled studies that investigated real-time PCR for this purpose, the authors detected 0.001% rendered bovine material when present in feed *(18)* and 1% bovine-derived material when present in meat-and-bone meal samples *(9).*

The determination of meat-and-bone meal concentrations in an unknown sample of feed or fish meal is performed by comparing the amplification of a target mtDNA sequence in an unknown sample with a calibration curve prepared with a known concentration of the same target in feed or fish meal.

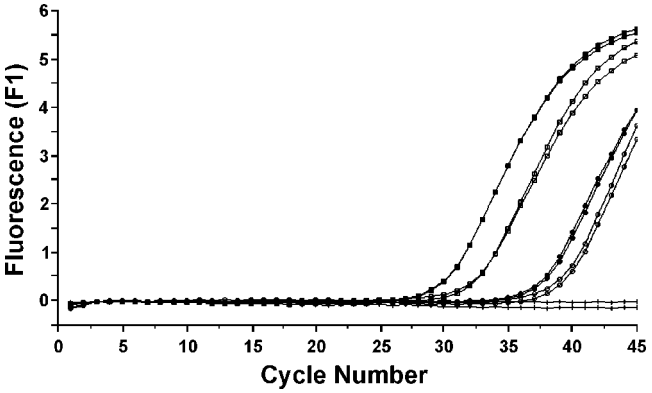

FIGURE 8. *Calibration curve for amplification of chicken DNA in fish meal containing 5 (■), 1 (□), 0.1 (●), and 0.01% (○) of bovine, ovine, swine, and chicken meat-and-bone meal, respectively; present in fish meal alone (|).*

It can be concluded that the method described in this article is suitable for the quantitative testing of feeds and fish meals for the presence of bovine, ovine, swine, and chicken meat-and-bone meals.

ACKNOWLEDGMENT

This work was supported by the Grant Agency of the Czech Republic (grant no. 525/01/0823).

REFERENCES

1. Anderson, S., M. H. L. de Bruijn, A. R. Coulson, I. C. Eperon, F. Sanger, and I. G. Young. 1982. Complete sequence of bovine mitochondrial DNA. Conserved features of the mammalian mitochondrial genome. *J. Mol. Biol.* 156:683–717.
2. Anonymous. 1994. Commission decision of 27 June 1994 concerning certain protection measures with regards to bovine spongiform encephalopathy and the feeding of mammalian derived protein (94/381/EC). *Off. J. Eur. Communities* L 172, p. 23–25.
3. Anonymous. 1998. Commission directive of 13 November 1998 establishing guidelines for the microscopic identification and estimation of constituents of animal origin for the official control of feedingstuffs (98/88/EC). *Off. J. Eur. Communities* L 318, p. 45–50.
4. Anonymous. 2001. Regulation of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (999/2001/EC). *Off. J. Eur. Communities* L 147, p. 1–40.
5. Ansfield, M., S. D. Reaney, and R. Jackman. 2000. Production of a sensitive immunoassay for detection of ruminant and porcine proteins, heated to $> 130^{\circ}C$ at 2.7 bar, in compound animal feedstuffs. *Food Agric. Immunol.* 12:273–284.
6. Bellagamba, F., V. M. Moretti, S. Comincini, and F. Valfre. 2001. Identification of species in animal feedstuffs by polymerase chain reaction-restriction fragment length polymorphism analysis of mitochondrial DNA. *J. Agric. Food Chem.* 49:3775–3781.
7. Bellagamba, F., F. Valfre, S. Panseri, and V. M. Moretti. 2003. Polymerase chain reaction–based analysis to detect terrestrial animal protein in fish meal. *J. Food Prot.* 66:682–685.
8. Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa. 1990. Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* 28: 495–503.
9. Brodmann, P. D., and D. Moor. 2003. Sensitive and semi-quantitative TaqMan™ real-time polymerase chain reaction systems for the detection of beef (*Bos taurus*) and the detection of the family *Mammalia* in food and feed. *Meat Sci.* 65:599–607.
10. Chen, F.-C., Y.-H. P. Hsieh, and R. C. Bridgman. 2004. Monoclonal antibody-based sandwich enzyme-linked immunosorbent assay for sensitive detection of prohibited ruminant proteins in feedstuffs. *J. Food Prot.* 67:544–549.
11. Colgan, S., L. O'Brien, M. Maher, N. Shilton, K. McDonnell, and S. Ward. 2001. Development of a DNA-based assay for species identification in meat and bone meal. *Food Res. Int.* 34:409–414.
12. Desjardins, P., and R. Morais. 1990. Sequence and gene organization of the chicken mitochondrial genome. A novel gene order in higher vertebrates. *J. Mol. Biol.* 212:599–634.
13. Frezza, D., M. Favaro, G. Vaccari, C. von Holst, V. Giambra, E. Anklam, D. Bove, P. A. Battaglia, U. Agrimi, G. Brambilla, P. Ajmonemarsan, and M. Tartaglia. 2003. A competitive polymerase chain reaction-based approach for the identification and semiquantification of mitochondrial DNA in differently heat-treated bovine meat and bone meal. *J. Food Prot.* 66:103–109.
14. Gizzi, G., L. W. D. van Raamsdonk, V. Baeten, I. Murray, G. Berben, G. Brambilla, and C. von Holst. 2003. An overview of tests for animal tissues in feeds applied in response to public health concerns regarding bovine spongiform encephalopathy. *Rev. Sci. Tech. Off. Int. Epiz.* 22:311–331.
15. Hiendleder, S., H. Lewalski, R. Wassmuth, and A. Janke. 1998. The complete mitochondrial DNA sequence of the domestic sheep (*Ovis aries*) and comparison with the other major ovine haplotype. *J. Mol. Evol.* 47:441–448.
16. Krcmar, P., and E. Rencova. 2001. Identification of bovine-specific DNA in feedstuffs. *J. Food Prot.* 64:117–119.
17. Krcmar, P., and E. Rencova. 2003. Identification of species-specific DNA in feedstuffs. *J. Agric. Food Chem.* 51:7655–7658.
18. Lahiff, S., M. Glennon, J. Lyng, T. Smith, N. Shilton, and M. Maher. 2002. Real-time polymerase chain reaction detection of bovine DNA in meat and bone meal samples. *J. Food Prot.* 65:1158–1165.
19. Myers, M. J., S. L. Friedman, D. E. Farrell, D. A. Dove-Pettit, M. F. Bucker, S. Kelly, S. Madzo, W. Campbell, R. F. Wang, D. Paine, and C. E. Cerniglia. 2001. Validation of a polymerase chain reaction method for the detection of rendered bovine-derived materials in feedstuffs. *J. Food Prot.* 64:564–566.
20. Myers, M. J., H. F. Yancy, and D. E. Farrell. 2003. Characterization of a polymerase chain reaction–based approach for the simultaneous detection of multiple animal-derived materials in animal feed. *J. Food Prot.* 66:1085–1089.
21. Tajima, K., O. Enishi, M. Amari, M. Mitsumori, H. Kajikawa, M. Kurihara, S. Yanai, H. Matsui, H. Yasue, T. Mitsuhashi, T. Kawashima, and M. Matsumoto. 2002. PCR detection of DNAs of animal origin in feed by primers based on sequences of short and long interspersed repetitive elements. *Biosci. Biotechnol. Biochem.* 66: 2247–2250.
22. Tartaglia, M., E. Saulle, S. Pestalozza, L. Morelli, G. Antonucci, and P. A. Battaglia. 1998. Detection of bovine mitochondrial DNA in ruminant feeds: a molecular approach to test for the presence of bovine-derived materials. *J. Food Prot.* 61:513–518.
23. Ursing, B. M., and U. Arnason. 1998. The complete mitochondrial DNA sequence of the pig (*Sus scrofa*). *J. Mol. Evol.* 47:302–306.
24. Wells, G. A. H., A. C. Scott, C. T. Johnson, R. F. Gunning, R. D. Hancock, M. Jeffrey, M. Dawson, and R. Bradley. 1987. A novel progressive spongiform encephalopathy in cattle. *Vet. Rec.* 121:419–420.