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Application of Quantitative Real-Time PCR in the Detection of Prion-Protein Gene Species-Specific DNA Sequences in Animal Meals and Feedstuffs

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ABSTRACT

This study describes a method for quantitative and species-specific detection of animal DNA from different species (cattle, sheep, goat, swine, and chicken) in animal feed and feed ingredients, including fish meals. A quantitative real-time PCR approach was carried out to characterize species-specific sequences based on the amplification of prion-protein sequence. Prion-protein species-specific primers and TaqMan probes were designed, and amplification protocols were optimized in order to discriminate the different species with short PCR amplicons. The real-time quantitative PCR approach was also compared to conventional species-specific PCR assays. The real-time quantitative assay allowed the detection of 10 pg of ruminant, swine, and poultry DNA extracted from meat samples processed at 130°C for 40 min, 200 kPa. The origin of analyzed animal meals was characterized by the quantitative estimation of ruminant, swine, and poultry DNA. The TaqMan assay was used to quantify ruminant DNA in feedstuffs with 0.1% of meat and bone meal. In conclusion, the proposed molecular approach allowed the detection of species-specific DNA in animal meals and feedstuffs.

The number of reported cases of bovine spongiform encephalopathy (BSE) in the European Union has reached a stationary phase in the spreading of the disease, and in some countries a significant decrease is apparent in the number of cases diagnosed between 2002 and 2005 *(7)*.

The first EU ban prohibiting the use of feedstuffs containing mammalian proteins was introduced in 1994 *(1)*. The ban and other measures subsequently introduced such as defining conditions of rendering (133°C, 20 min, 300 kPa) and the designating specific risk material have played a critical role in the BSE eradication program. The theory of feedstuff contamination through contamination of animal proteins with abnormal prion proteins has been supported by epidemiological and rendering studies and, recently, by the evaluation of the effect of this feed ban on the decrease in the BSE cases *(7)*.

After the 1994 ban, the European Union further limited the use of animal by-products in animal nutrition *(3–5)*. In part, because of the lack of sensitive and reliable methods for determining the presence of prohibited mammalian protein in feed, several amendments to the 1994 legislation were introduced *(3–5)*. The final decision adopted in 2000 *(5)* banned the feeding of processed animal protein to farmed animals reared, fattened, or bred for the production of food, with the exception of the fish meal to nonruminant species. The above-mentioned decision is a clear demonstration that, in the absence of adequate control measures avoiding the presence of prohibited material in animal feed, the total ban was necessary to prevent further spread of BSE. The development and validation of laboratory techniques for detecting and quantifying ruminant proteins could allow for verifying the compliance with regulations that could lead to various amendments of the current regulations. There are critical conditions that must be respected to avoid the risk of contamination with infected material *(23)*, such as the need to avoid feed cross-contamination with prohibited material and intraspecies recycling. In order to control and/or exclude the presence of fallen livestock and other suspect material from the feed chain, a definitive European regulation established further rules concerning animal by-products *(6)*. The conditions set in this directive consolidate previous legislation and introduce new features, like the rule that only materials from animals declared by veterinary inspection as safe for human consumption might be used in the production of feed, as well as the enforcement of the ban towards intraspecies recycling.

Considering that only a zero level of cross-contamination would exclude any associated risk and that this condition is not practically enforceable given the absence of species-dedicated feed mills that manufacture feed for a single species only (with the exception for the production of feedstuff for fish), the differentiation between animal proteins from different origins needs to be based on a validated analytical method.

The microscopic method was validated in 1998 by an intercomparison study and became the official assay available for the determination of meat and bone meal (MBM) in feedstuff *(2)*. With the aim to enforce EU regulations

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concerning the ban of feeding MBM to mammalian and the intraspecies recycling, several methods have been developed for the analysis of feed *(8–10, 14, 16, 18, 20, 22, 24)*. However, no test is definitive in fulfilling all the testing requirements *(15)*. For instance, the performance of the microscopic method is largely independent of the rendering conditions under which the material was processed, i.e., bone is unaffected by rendering; however, the sensitivity depends on the experience of the operator. Furthermore, microscopy cannot differentiate between mammalian species. On the other end, the immunochemical and molecular techniques are largely capable of differentiating animal species, but their performances are dependent on rendering conditions used at manufacture of animal materials. Some believe that immunoassays and spectroscopy may be applied as screening methods, whereas PCR and, in some cases, the microscopic methods may be used for more specific analytical purposes. Recently, quantitative real-time PCR assays with specific fluorogenic TaqMan-based probes have been described *(12, 17, 19, 21)*. Among the advantages achieved by these techniques is the detection of small-size DNA amplified fragments (from 66 to 145 bp), which means that it is possible to characterize DNA from highly degraded sources, like rendered MBM. In this study, we describe a specific detection of animal DNA from different species in animal protein meals samples (including fish meals), applying a quantitative real-time PCR approach. The TaqMan-based assays were designed to amplify species-specific prion-protein (PRNP) gene sequences; these assays enabled the direct quantification of the DNA content of a single animal species within feedstuff. Finally, in order to verify the feasibility to apply the PCR method in the quantitative detection of ruminant material in feedstuffs, purposely contaminated feedstuffs with known amount of MBM were analyzed.

MATERIALS AND METHODS

Samples and DNA extraction. Single-species laboratory-prepared meat meals (LPMMs) were obtained under controlled temperature conditions as previously described *(11)*. Briefly, raw tissues, including offal and bone material, from cattle (*Bos taurus*), sheep (*Ovis aries*), goat (*Capra hircus*), swine (*Sus scrofa*), and chicken (*Gallus gallus*) were exposed to sterilization heat treatment (130°C, 40 min, 200 kPa) to reproduce the DNA degradation which was verified in MBM after rendering process. MBM, blood meal (BM), and meat meal (MM) samples were purchased from different rendering plants in Italy. Fish meal (FM) samples were obtained from the market. Sample feedstuffs containing a known percentage of MBM (0.1, 0.5, and 2%) were kindly donated by Dr. von Holst (Institute for Reference Materials and Measurements, Joint Research Centre, Geel, Belgium). In addition, genomic DNA from peripheral blood cells of different species (cattle, sheep, goat, swine, horse, chicken, turkey, buffalo, and rabbit) was isolated by using a commercial kit (Qiagen Inc., Valencia, Calif.).

DNA was isolated from LPMMs, MBM, BM, MM, FM, and sample feedstuffs as previously described *(11)*. In brief, a 2-g aliquot from each sample was digested with 400 μl of proteinase K (20 mg/ml; Sigma, St. Louis, Mo.) in 8.6 ml of lysis buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% sodium dodecyl sulfate), containing 1 ml 5 M guanidine hydrochloride. After 4 h at 57°C, the digestion was stopped, and the aqueous supernatant obtained by centrifugation (10,000 × *g* for 20 min) was separated. The supernatant phase was added to an equal volume of Wizard DNA purification resin in a Wizard Midicolumn (Promega, Madison, Wis.), and the DNA was isolated according to the manufacturer's instructions. The purified DNA was stored in 300 μl of nuclease-free water at −20°C. DNA concentration was accurately quantified with spectrophotometric analyses (BioPhotometer, Eppendorf, Hamburg, Germany).

Primers and probes. TaqMan primers and internal specific minor groove-binding (MGB) probes were designed (Italian patent no. MI2004A000991) within the PRNP coding sequences by means of the Primer Express software (version 3.1, Applied Biosystems, Foster City, Calif.) with identical annealing temperatures (70 and 59°C for the primers and the MGB probes, respectively). Primers were designed on the PRNP sequences of the following species: *Bos taurus* (GenBank entry AJ298878), *Capra hircus* (S82626), *Ovis aries* (AJ223072), *Sus scrofa* (L07623), and *Gallus gallus* (M95404).

A ruminant-specific (bovine, ovine, caprine) TaqMan assay was designed to detect simultaneously DNA from cattle, sheep, and goat. This was performed on PRNP consensus sequence with the primers 5′-GGCCATGTGGAGTGACGTG and 5′-CATCC TCCTCCAGGTTTTGGT and probe 5′–6-FAM-CCTCTGCAA GAAGC-(MGB). Differently, a swine-specific TaqMan assay was performed using the primers 5′-TTTGTGCATGACTGCGTC AAC and 5′-CTTGGTGGTCGTGGTCACTGT and probe 5′–6-FAM-CACCGTCAAGCAGC-(MGB). A chicken-specific TaqMan assay was designed using the primers 5′-CGGGTTTACTACCGG GATTACA and 5′-GCAATCGGCCACGAAGAC and probe 5′–6-FAM-CCCGTGCCACAGGA-(MGB). TaqMan probes were synthesized by Applied Biosystems.

The optimal concentration was experimentally decided to be 0.9 μM for all primers and 0.2 μM for all probes.

Quantitative and qualitative PCR amplification. Quantitative real-time PCR was performed on a TaqMan 5700 instrument (Applied Biosystems) in a total volume of 25 μl containing extracted DNA (8 to 2 ng), the primers (0.9 μM), the probes (0.2 μM) and 1× TaqMan Universal PCR Master Mix, No AmpErase UNG (product no. 4324018, Applied Biosystems). The PCR program included 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 59°C.

The real-time fluorescent detection was based on the hydrolysis of the internal labeled TaqMan probe which increases the specificity of the assay compared to conventional qualitative PCR assembled with a couple of primers. Fluorescent emission, generated by the hydrolysis of the labeled internal probe, is detected and measured during the reaction and is directly proportional to the amount of template amplification obtained. The end point of fluorescence detection is expressed as the cycle threshold (Ct) value, which is inversely proportional to the number of target DNA template copies that are present in the reaction tube; by plotting the Ct values against the log of known amounts of target DNA, a standard curve is obtained and the quantification of target DNA in unknown samples is then recovered by interpolation of Ct values into this curve.

In order to demonstrate the presence of ruminant (bovine, ovine, caprine), porcine, and poultry DNA in animal meal samples (MBM, BM, MM, and FM), conventional qualitative PCR amplification of mitochondrial specific region of 12S ribosomal RNA gene was carried out as previously reported *(11)*.

TABLE 1. *Cycle threshold (Ct) values[a] obtained from four replicate analyses with real-time species-specific PCR of known amounts of DNA extracted from single-species laboratory-prepared meat meals*

Quantity of DNA (ng)	Ruminant probe		Porcine probe		Poultry probe	
	Ct	RSD (%)[b]	Ct	RSD (%)	Ct	RSD (%)
0.5	29.68 ± 0.30	1.0	30.84 ± 0.18	0.58	28.43 ± 0.19	0.67
5	26.51 ± 0.42	1.6	24.91 ± 0.22	0.88	24.87 ± 0.21	0.84
10	25.75 ± 0.24	0.3	23.03 ± 0.18	0.78	23.21 ± 0.64	2.75
50	23.31 ± 0.48	2.0	22.93 ± 0.06	0.26	21.48 ± 0.33	1.53
100	22.66 ± 0.32	1.4	22.22 ± 0.21	0.94	20.43 ± 0.20	0.98

[a] $n = 4$.
[b] RSD, relative standard deviation.

RESULTS

The specificity of each probe was confirmed by real-time PCR amplification of genomic DNA extracted from peripheral blood of different species (cattle, sheep, goat, buffalo, horse, swine, turkey, rabbit, and chicken). No species cross-reactivity was found with TaqMan assays (data not shown).

Standard curves were produced for each species-specific PCR assay by using degraded DNA obtained from LPMMs. The corresponding Ct values of fivefold dilutions (0.5, 2.5, 5, 25, and 50 ng) of ruminant (equal amounts of cattle, sheep, and goat DNA), swine, and chicken DNA from single-species MM analyzed in four replicates are reported in Table 1. The standard curve was obtained by plotting the mean Ct versus the logarithm of the DNA concentration (Fig. 1); the linearity was expressed as R^2.

In Table 2, quantitative real-time PCR results obtained from animal meal samples are reported. Quantitative DNA estimation expressed in nanograms was performed on the basis of the standard curves obtained for each specific probe; these values represent the haploid DNA content, assuming that the PRNP sequence target is a single copy gene, as previously documented *(13).* The percentage of ruminant (bovine, ovine, caprine), porcine, and poultry DNA (Table 3) was related to the total DNA amount extracted from each samples.

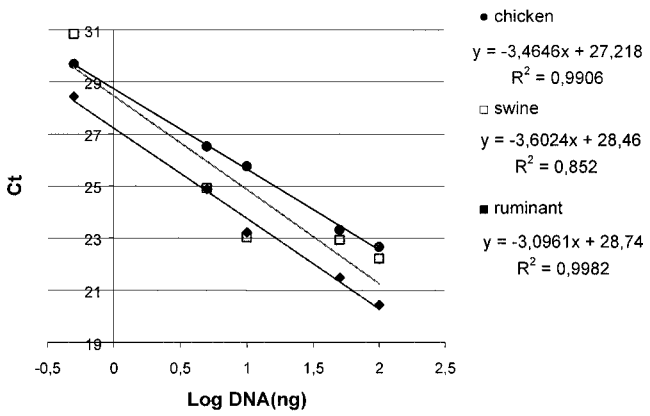

FIGURE 1. *Regression data obtained by plotting cycle threshold (Ct) values versus the logarithm of known different concentrations of chicken, swine, and ruminant (cattle, sheep, goat) DNA extracted from single-species laboratory-prepared meat meals (LPMMs).*

The described quantitative real-time PCR assay was also carried out in order to verify the detection of ruminant DNA in experimental feedstuffs containing 0.1, 0.5, and 2% of MBM. The application of real-time PCR method allowed the detection of ruminant (bovine, ovine, caprine) DNA material in experimental feedstuffs (Table 4).

Subsequently, in order to confirm the presence and/or the absence of ruminant (bovine, ovine, caprine), porcine, and poultry DNA in analyzed animal meals, conventional qualitative PCR assay was used to amplify mitochondrial specific target sequence of the 12S ribosomal RNA gene (Fig. 2) by using previously described conditions *(11).* The obtained species-specific PCR products confirmed the results obtained in the real-time PCR analyses (Table 3).

DISCUSSION

The observed relationship between BSE and ruminants eating feeds containing animal tissues contaminated with the agent of transmissible spongiform encephalopathies (TSE) has resulted in numerous efforts to develop and validate methods for detection of prohibited animal material in feedstuffs. To avoid the risk of unintentional contamination with prohibited ruminant material in the preparation of MBM for animal feed, the EU legislation imposed restrictive rules concerning the introduction of animal by-products in the feeding of farmed species. The real-time PCR methods developed to characterize the origin of animal protein are mainly aimed at reaching an adequate sensitivity in the detection of very low levels of degraded DNA from banned species and a high species specificity. Because ruminant species, especially bovine and ovine, represent the main concern and possible source of TSE epidemic, most of the assays have been focused on the detection and quantification of biological traces of these species. However, considering that a recent EU directive *(6)* has put much more attention on the control of and the risk of intraspecies recycling, it becomes imperative to extend the analysis to the identification of several domestic animal species within animal feedstuffs.

In order to detect the presence of ruminant (bovine, ovine, caprine), porcine, and poultry material in animal protein meals, we have developed a real-time PCR assay able to quantify DNA originated from these species at a level that falls within the range of 10 pg to 100 ng.

The analytical procedure described in this study was

TABLE 2. *Cycle threshold (Ct) values[a] obtained from real-time PCR for detection of ruminant (bovine, ovine, caprine), porcine, and poultry DNA extracted from meat meal, blood meal, meat and bone meal, and fish meal samples[b]*

	Ruminant probe		Porcine probe		Poultry probe	
Sample[c]	Ct	DNA (ng)	Ct	DNA (ng)	Ct	DNA (ng)
MM	27.47 ± 0.43	2.6	22.9 ± 1.32	34.67	ND[d]	ND
BM	26.85 ± 0.41	4.0	21.8 ± 1.40	70.8	ND	ND
MBM 1	24.94 ± 0.33	16.6	24.01 ± 0.27	17	27.63 ± 0.55	0.78
MBM 2	26.94 ± 1.57	3.8	24.56 ± 0.36	12.02	28.75 ± 1.17	0.37
MBM 3	25.4 ± 1.48	12.0	25.03 ± 0.4	8.9	28.13 ± 0.78	0.55
MBM 4	29.24 ± 1.88	0.7	23.77 ± 0.39	19.95	21.38 ± 1.27	46.77
MBM 5	24.46 ± 0.35	24.0	21.85 ± 0.37	67.6	25.86 ± 0.61	2.45
FM 1	ND	ND	ND	ND	ND	ND
FM 2	ND	ND	ND	ND	ND	ND
FM 3	ND	ND	ND	ND	ND	ND
FM 4	ND	ND	ND	ND	ND	ND

[a] $n = 4$.
[b] Absolute quantitative estimation of specific DNA was calculated from the calibration curves obtained for each species-specific probe and is reported in Figure 1.
[c] MM, meat meal; BM, blood meal; MBM, meat and bone meal; FM, fish meal.
[d] ND, not detected; a Ct value of 40 was measured with no amplification signal after 40 cycles.

based on detecting species-specific portions of the PRNP coding gene. PRNP coding sequences are available in nucleotide databases for several species. Furthermore, the gene is well conserved across vertebrates, allows identification of conserved regions between different species (i.e., cattle, sheep, and goat), and exhibits several interspecific or species-specific nucleotide variations. Such differences were exploited in our approach to design species-specific TaqMan-based probes.

To partially overcome the sensitivity limit, we adopted high-stringent TaqMan-based probes, namely the TaqMan minor groove binding (MGB); in addition, optimal PCR conditions were developed after accurate kinetic studies. Furthermore, MGB probes, coupled with sequence-specific primers, enabled the amplification of very short amplicons ranging from 57 to 60 nucleotides. Similarly, Brodmann and Moor *(12)* approached a semiquantitative PCR reaction to amplify growth hormone gene DNA fragments, ranging from 66 to 76 nucleotides, to detect mammalian DNA in food and MBM.

Because it is well known that degradation of the DNA can significantly compromise the sensitivity of the assay, in order to quantify degraded DNA from animal meals, we considered producing a calibration curve using DNA extracted from processed meat, but it was not ignored that DNA levels in rendered materials are significantly related to rendering conditions. The proposed real-time–based analytical approach turned out to be sensitive and specific as documented by the linear response (Fig. 1) obtained in the calibration curves for ruminants (bovine, ovine, caprine) ($R^2 = 0.998$) and chicken ($R^2 = 0.990$); whereas, for pig, a lower value ($R^2 = 0.852$) of linear regression coefficient

TABLE 3. *Results obtained from conventional qualitative species-specific PCR and quantitative specific real-time PCR[a]*

		Ruminant probe		Porcine probe		Poultry probe	
Sample[b]	DNA yielded (ng/μl)	Qualitative PCR	Real-time PCR, ruminant DNA (%)	Qualitative PCR	Real-time PCR, porcine DNA (%)	Qualitative PCR	Real-time PCR, poultry DNA (%)
MM	83	Positive	3.1	Positive	41.8	Negative	ND[c]
BM	100	Positive	4.1	Positive	70.8	Negative	ND
MBM 1	110	Positive	15.1	Positive	15.4	Positive	0.7
MBM 2	87	Positive	4.4	Positive	13.8	Positive	0.4
MBM 3	22.4	Positive	53.7	Positive	39.7	Positive	2.4
MBM 4	89	Positive	0.8	Positive	22.4	Positive	52.5
MBM 5	97	Positive	24.7	Positive	69.7	Positive	2.5
FM 1	115	Negative	ND	Negative	ND	Negative	ND
FM 2	96	Negative	ND	Negative	ND	Negative	ND
FM 3	102	Negative	ND	Negative	ND	Negative	ND
FM 4	98	Negative	ND	Negative	ND	Negative	ND

[a] Amounts of species-specific DNA are expressed as percentages to total DNA yielded.
[b] MM, meat meal; BM, blood meal; MBM, meat and bone meal; FM, fish meal.
[c] ND, not detected; a Ct value of 40 was measured with no amplification signal after 40 cycles.

TABLE 4. *Cycle threshold (Ct) values obtained from real-time PCR with ruminant (bovine, ovine, caprine) probes on DNA extracted from feedstuff samples*

	Ruminant probe			
Sample[a]	Ct ($n = 2$)	Detected ruminant DNA (ng)[b]	DNA yield from extraction (ng/μl)	Ruminant DNA (%)[c]
0.01 ng	34.21 ± 0.43			
0.05 ng	33.70 ± 0.65			
0.1 ng	28.90 ± 0.80			
0.5 ng	29.29 ± 0.47			
5 ng	26.25 ± 0.22			
50 ng	23.13 ± 0.06			
100 ng	22.99 ± 0.32			
Feedstuffs				
0.1% MBM	28.10 ± 0.52	1.14	974.4	0.11
0.5% MBM	27.25 ± 0.62	2.29	711	0.32
2% MBM	24.66 ± 0.50	19.5	931	2.09

[a] MBM, meat and bone meal.
[b] Amounts of ruminant DNA were estimated from the calibration curve reported.
[c] Percentages of ruminant DNA were calculated based on the total amount of DNA extracted.

was obtained, probably due to a lower amplification efficiency. The sensitivity level of 0.5 ng obtained in the porcine and poultry DNA calibration curve (Table 1) does not reflect the actual limit of detection of the PCR system, but that the values between 100 and 0.5 ng were considered the optimal range for the DNA detection of these species in the analyzed animal meal samples. In different trials carried out in our laboratory applying the described TaqMan assays for chicken and swine DNA detection, we verified the linearity of both methods using significantly lower (20 to 50 pg) DNA concentrations (data not shown).

The estimation of species-specific DNA in animal meal samples was calculated with an absolute quantification (Table 2). In Table 3, we reported the percentage of ruminant (bovine, ovine, caprine), porcine, and poultry DNA related to the total amounts of DNA in the sample. The species-specific DNA detection and characterization using the quantitative real-time PCR approach is in complete agreement with the results obtained by applying conventional PCR and species-specific primers; no false-positive results were shown when crossing specific TaqMan-MGB probes against other no-target species. Furthermore, the absence of amplification curves in fish meal samples excluded the presence of detectable quantities of ruminant, swine, and chicken in these samples (Table 3).

In order to quantify ruminant DNA in feedstuffs adulterated with MBM at different levels (0.1, 0.5, and 2%), we provided a different calibration curve, ranging from 100 ng to 10 pg, with ruminant (bovine, ovine, caprine) degraded DNA from single species LPMMs (Table 4); the obtained linear regression analysis showed a significant linearity ($R^2 = 0.933$). Although we agree that the detection of DNA sequences of a single copy gene decreases the sensitivity of the method when compared with the use of mitochondrial DNA *(17, 19)* or repetitive elements *(21)*, the described real-time PCR assay based on targeting a nonrepetitive genomic sequence allowed the detection of 10 pg of ruminant (bovine, ovine, caprine) material in single-species LPMMs exposed to heat treatment (130°C, 40 min, 200 kPa) (Table 4). The TaqMan assay allowed the detection of 1.14 ng of ruminant DNA in feedstuffs with 0.1% of MBM (Table 4).

Although a more accurate strategy for DNA quantification approaching a relative quantification with the simultaneously real-time amplification with different housekeeping gene should be carried out, the proposed absolute quan-

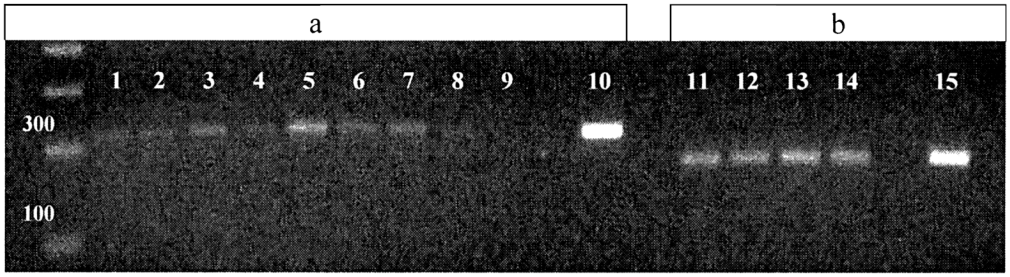

FIGURE 2. *Conventional PCR amplification results. (a) 231 bp species-specific ruminant region of the 12S mitochondrial DNA; lanes 1 through 5, meat and bone meal (MBM) samples; lanes 6 through 8, feedstuffs containing 2, 0.5, and 0.1% of MBM; lane 9, fish meal (FM); lane 10, positive control bovine genomic DNA; (b) 186 bp species-specific porcine region of the 12S mitochondrial DNA; lanes 11 through 14, MBM samples; lane 15, positive control porcine genomic DNA.*

tification based on the generation of a calibration curve could represent a starting point in developing multiplex-based PCR amplification systems.

The above-described procedure might be highly adaptable to any species-specific DNA monitoring program, particularly those related to the animal and human food chain. In fact, the species potentially detectable as well as the sensitivity of the detection systems are likely to increase, owing to the continuous flow of DNA sequences deposited in public repositories and to the technical advances in real time assays. Moreover, several efforts should be made to investigate and verify the response of the described methods in relation to the different levels of DNA degradation obtained in rendered materials.

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REFERENCES

1. Anonymous. 1994. Commission Decision 94/381/EC, p. 23–24. *Off. J. Eur. Commun.* L172:27 June 1994.
2. Anonymous. 1998. Commission Decision 1998/88/EC, p. 45–50. *Off. J. Eur. Commun.* L318:27 November 1998.
3. Anonymous. 1999. Council Decision 99/534/EC, p. 37–42. *Off. J. Eur. Commun.* L204:4 August 1999.
4. Anonymous. 2000. Commission Decision 2000/418/EC, p. 76–24. *Off. J. Eur. Commun.* L158:30 June 2000.
5. Anonymous. 2000. Council Decision 2000/766/EC, p. 32–33. *Off. J. Eur. Commun.* L 306:7 December 2000.
6. Anonymous. 2002. Regulation 2002/1774/EC, p. 1–95. *Off. J. Eur. Commun.* 273:10 October 2002.
7. Anonymous. 13 June 2005. Number of reported cases of bovine spongiform encephalopathy (BSE) in farmed cattle worldwide. Available at: http://www.oie.int/eng/info/en_esbmonde.htm. Accessed 16 June 2005.
8. Ansfield, M., S. D. Reaney, and R. Jackman. 2000. Production of a sensitive immunoassay for detection of ruminant and porcine proteins, heated to >130°C at 2.7 bar, in compound animal feedstuffs. *Food and Agric. Immunol.* 12:273–284.
9. Baeten V., C. von Holst, A. Garrido, J. Vancutsem, A. Michotte Renier, and P. Dardenne. 2005. Detection of banned meat and bone meal in feedstuffs by near-infrared microscopic analysis of the dense sediment fraction. *Anal Bioanal. Chem.* 382:149–157.
10. Bellagamba, F., V. M., Moretti, S. Comincini, and F. Valfrè. 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.
11. Bellagamba, F., F. Valfrè, S. Panseri, and V. M. Moretti. 2003. Polymerase chain reaction–based analysis to detect terrestrial animal protein in fish meal. *J. Food Prot.* 66:513–518.
12. 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.
13. Castiglioni, B., S. Comincini, B. Drisaldi, T. Mottam, and L. Ferretti. 1998. Comparative mapping of the prion gene (PRNP) locus in cattle, sheep and human with PCR-generated probes. *Mamm. Genome* 10: 853–855.
14. Gao, H. W., D. B. Zang, A. H. Pan, W. Q. Liang, and C. Z. Liang. 2003. Multiplex polymerase chain reaction method for detection of bovine materials in foodstuffs. *J. AOAC Int.* 86:764–767.
15. 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.
16. Krcmar, P., and E. Rencova. 2003. Identification of species-specific DNA in feedstuffs. *J. Agric. Food Chem.* 51:7655–7658.
17. Krcmar, P., and E. Rencova. 2005. Quantitative detection of species-specific DNA in feedstuffs and fish meals. *J. Food Prot.* 68:1217–1221.
18. Kusama, T., T. Nomura, K., and K. Kadowaki. 2004. Development of primers for detection of meat and bone meal in ruminant feed and identification of the animal origin. *J. Food Prot.* 67:1289–1292.
19. Lahiff, S., M. Glennon, J. Lyng, T. Smith, N. Shilton, and M. Maher. 2002. Real-time polymerase chain reaction of bovine DNA in meat and bone meal samples. *J. Food Prot.* 65:1158–1165.
20. Lahiff, S., M. Glennon, L. O'Brien, J. Lyng, T. Smith, M. Maher, and N. Shilton. 2001. Species-specific PCR for the identification of ovine, porcine and chicken species in meat and bone meal. *Mol. Cel. Probes* 15:27–35.
21. Mendoza-Romero, L., E. L. C. Verkaar, P. H. Savelkoul, A. Catsburg, H. J. M. Aarts, J. B. Buntjer, and J. A. Lenstra. 2004. Real-time detection of ruminant DNA. *J. Food Prot.* 67:550–554.
22. Myers, M. J., S. L. Friedman, D. E. Farrel, D. A. Dove-Pettit, M. F. Bucker, S. Kelly, S. Madzo, W. Campbell, R. Wang, D. Paine, and C. E. Carniglia. 2001 Validation of a polymerase chain reaction method for the detection of rendered bovine-derived materials in feedstuffs. *J. Food Prot.* 64:564–566.
23. Scientific Steering Committee (SSC). 2000. Opinion on the scientific basis for banning animal protein from the feed for all farmed animals, including pig, poultry, fish and pet animals. Adopted at the SSC meeting of 27 to 28 November 2000. Available at: http://www.europa.eu.int/comm/food/fs/sc/ssc/out150_en.pdf. Accessed 16 June 2005.
24. Toyoda, A., M. Nakajo, H. Kawachi, T. Matsui, and A. Yano. 2004. PCR detection of bovine mitochondrial DNA derived from meat and bone meal in feed. *J. Food Prot.* 67:2829–2832.