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Detection of Bovine Mitochondrial DNA in Ruminant Feeds: A Molecular Approach to Test for the Presence of Bovine-Derived Materials

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ABSTRACT

A ban on ruminant-derived proteins in ruminant feeds has been introduced as a preventive measure to avoid the spread of bovine spongiform encephalopathy (BSE), as well as to minimize any potential risk of BSE transmission from bovines to humans. In the absence of commercially available efficient methods for identification of bovine-derived proteins in animal feeds, we developed a rapid and sensitive polymerase chain reaction (PCR)-based assay which allows detection and identification of a bovine-specific mitochondrial DNA sequence from feedstuffs. The amplified product encodes for the whole ATPase subunit 8 and the amino-terminal portion of the ATPase subunit 6 proteins, which are known to exhibit a relatively low degree of conservation among vertebrates. The specific amplification of such a bovine mitochondrial sequence from reference feedstuff samples was demonstrated by means of both direct sequencing and single-strand conformational analysis of the PCR product. Specificity was also confirmed by the absence of detectable homologous PCR product when using reference feedstuff samples lacking bovine-derived meat and bonemeals, or genomic DNA samples from vertebrates whose offals are commonly included in animal feeds. This method allows detection of the presence of bovine mitochondrial DNA in feedstuffs containing less than 0.125% of bovine-derived meat and bonemeals. Furthermore, it does not appear to be considerably affected by prolonged heat treatment. *Dpn*II and *Ssp*I restriction endonuclease digestions of the unpurified PCR product may be used routinely to confirm the bovine origin of the amplified sequence. Since this method is specific, rapid, and sensitive, it could be successfully utilized as a routine control assay to evaluate the presence of bovine-derived meat and bonemeals in ruminant feeds.

Bovine spongiform encephalopathy (BSE) is a fatal degenerative disease affecting the central nervous system of cattle *(27)*. BSE was first recognized in Great Britain in 1986, and its incidence progressively increased until 1993, with more than 100,000 cattle being affected *(1)*. According to Wilesmith et al. *(28)*, the BSE epidemic arose from feeding cattle with rendered protein supplements derived from scrapie-infected sheep tissues, and its spread was strictly associated with the absence of stringent controls on rendering processes. Since 1988, a ban on enriching cattle feeds with ruminant-derived proteins has led to a decline in the incidence of BSE in Great Britain. Nevertheless, several cases have been observed in other European countries in the last few years, and at least 15 cases of a new variant of the Creutzfeldt-Jakob disease (nvCJD), which is supposed to be etiologically linked to BSE, have been reported in Great Britain and France *(8, 24)*. At present, a ban on feeding ruminants with ruminant-derived proteins is in force in European countries and the United States. Such a prohibition seems to be the most effective preventive measure to avoid the spread of BSE or other transmissible spongiform encephalopathies (TSE), as well as to minimize any potential risk of BSE transmission to humans. In the light of such a ban, immunological assays and microscopic examination methods have been developed to test for the presence of ruminant or mammalian proteins respectively in animal feedstuffs. However, the failure of commercially available immunoassays, due to protein heat denaturation associated with the rendering process, and the low resolution of the microscopic method, which allows detection exclusively of mammalian, but not species-specific bone fragments, testify to the need of more reliable control assays.

Recombinant DNA technology allows a new and alternative approach. The polymerase chain reaction (PCR) leads to an in vitro amplification of specific target DNA sequences by using the appropriate oligonucleotide primer pairs *(9, 14)*. This technique offers a high level of sensitivity, permitting a million-fold increase of the starting template fragment. Furthermore, an optimized PCR procedure successfully amplifies the specific target sequence even in a very complex pool of genomic sequences. Because of these real advantages, numerous PCR-based methods for detection of pathogens in food as well as for the identification of species composition in food products have been developed in the last few years *(12, 15)*.

Here we report a method that, coupling the specificity and sensitivity of the polymerase chain reaction and the efficiency of a silica-based nucleic acid extraction procedure, allows rapid detection and identification of a bovine-specific mitochondrial DNA (mtDNA) sequence from ani-

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mal feeds, as a marker for the presence of bovine-derived tissues in feedstuffs. Such a PCR-based method exhibits high specificity, sensitivity, and rapidity, and in our opinion it could be successfully utilized as a routine control assay to evaluate the addition of bovine-derived meat and bonemeals in ruminant feeds.

MATERIALS AND METHODS

Samples and DNA extraction. Reference feedstuffs were purposely produced (Laboratorio Sperimentale di Produzione di Alimenti per Animali, Facoltà di Agraria, Università Cattolica del Sacro Cuore, Piacenza, Italy), as enacted by law. A detailed description of the rendering process is reported in *(3)*. Reference feedstuffs included bovine-derived meat and bonemeal (MBM)-free samples, as negative controls, and samples containing 2, 1, 0.5, 0.25, or 0.125% of MBM, which were made up of 70% bovine, 20% porcine, and 10% poultry materials, with negligible amounts of sheep-, rabbit- and fish-derived material. One hundred percent bovine MBM and fish meal (FM) samples were also respectively included in the study as positive and negative controls.

Total DNA was extracted from feedstuff samples according to the method described by Boom et al. *(4)*, with minor modifications. This method is based on the DNA-binding property of silica particles in the presence of guanidinium thiocyanate (GuSCN), the latter being preferred to other chaotropic agents because of its nuclease-inactivating action. All chemicals utilized were of molecular biology grade, and unless otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO). The extraction procedure was optimized as follows. Extraction samples consisted of 0.5 g of feedstuff pellet ground to a fine powder. Each sample was added to 4 to 5 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). It was vortexed and incubated at 60°C in a water bath. The efficiency of DNA extraction appeared to be strictly related with the time of sample incubation. Best results were obtained with overnight incubation (15 to 20 h). Successively, samples were vortexed, centrifuged (2,500 × *g*, 5 min), and about 500 μl of the supernatant was added to a mixture including 500 μl of extraction buffer and 40 μl of silica suspension, the latter being prepared as described in Boom et al. *(4)*. The mixture was vortexed, incubated at room temperature (10 min), centrifuged in an Eppendorf microfuge (13,000 rpm, 1 min), and the supernatant was carefully removed by suction. The silica pellet was subsequently washed twice with washing buffer (5 M GuSCN; 0.05 M Tris-HCl, pH 6.4), twice with 70% ethanol, and once with acetone. The tube was dried at 56°C with open lid in a heat block for 10 min. Total DNA was subsequently eluted at 56°C in one aliquot of 50 μl of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0), and stored at −20°C until analyzed. As previously reported *(13)*, we observed that even minimal amounts of silica particles were able to inhibit the PCR. For this reason, a second centrifugation (13,000 rpm, 2 min) of the eluted sample was performed.

To prevent DNA contamination, extractions were carried out with dedicated equipment in a laminar flow hood, in a dedicated laboratory room. PCR preparations and post-PCR analyses were performed in separate rooms. Both negative controls, lacking DNA or a bovine DNA source, and positive controls, consisting of bovine-derived MBM and bovine genomic DNA, were included in each extraction set. Pipettors were sterilized between use by UV irradiation, and aerosol-resistant plugged pipette tips (ART®, Molecular Bio-Product, Inc., CA) were utilized during both DNA extraction and PCR preparation. Multiple independent DNA extractions were carried out from each sample.

Genomic DNAs from cow, sheep, swine, horse, rabbit, chicken, and turkey were extracted from peripheral blood leucocytes by means of the salting-out method *(18)*.

PCR procedure. A single PCR round was performed using the following primers: Forward (L-strand), L8129 5′-GCCATATACTCTCCTTGGTGACA-3′; reverse (H-strand), H8357 5′-GTAGGCTTGGGAATAGTACGA-3′. This primer pair amplifies a 271-bp bovine mitochondrial fragment encoding for the 3′-portion of $tRNA^{Lys}$, the ATPase subunit 8 (ATPase8) and the amino-terminal portion of the ATPase subunit 6 (ATPase6) *(2)*. Amplifications were carried out by using a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) in 50 μl of reaction containing 12.5 pmol of each primer, 50 μM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% (wt/vol) gelatin, 1.5 mM $MgCl_2$ and 2 U of Ampli*Taq* DNA polymerase (Roche Molecular System Inc., Branchburg, NJ). As template, 2 to 5 μl of DNA extract from feedstuff samples or 200 ng of genomic DNA was used. After an initial denaturation step at 94°C for 60 s, PCR conditions were optimized as follows: 30 cycles at 94°C, 60 s; 58°C, 30 s; 72°C, 30 s (last extension step: 72°C, 5 min). PCR negative and positive controls were also included in parallel with each amplification set. Sequences of primer pairs utilized to amplify fragments including the $tRNA^{Lys}$-ATPase8-ATPase6 homologous sequences from sheep, swine, horse, and chicken genome DNAs are available upon request.

PCR product analyses. In order to confirm the amplification of the target sequence and the bovine specificity of the L8129/H8357 oligonucleotide pair, 0.5 pmol of purified products from a second round PCR (QIAEX II Extraction Kit, QIAGEN, Hilden, Germany) were directly sequenced on both strands according to Casanova *(7)* by using the Sequenase Kit version 2.0 (United States Biochemicals, Cleveland, OH) and, as primers, the same oligonucleotides utilized during PCR. Bovine-specific amplification from feedstuff samples was also evaluated by means of single-strand conformational analysis (SSCA) *(19, 20)* and *Ssp*I and *Dpn*II endonuclease digestions. Briefly, SSCA was carried out by denaturing 4 μl of [α-^{35}S]dATP-labeled PCR products in an equal volume of a 95% formamide, 20 mM EDTA solution, to 94°C for 5 min. Samples were immediately cooled at −80°C for 10 min, put on ice and loaded on a 5% and 6% acrylamide (ratio 29:1; Bio-Rad Laboratories, Hercules, CA) gel containing 0.5% and 5% glycerol, respectively. *Ssp*I and *Dpn*II endonuclease digestions of both purified and unpurified PCR products were performed in a 20 to 35 μl reaction volume containing 3 to 5 U of enzyme and 5 to 10 μl of PCR extract, according to the manufacturer's specifications (New England Biolabs Inc., Beverly, MA). *Ssp*I and *Dpn*II restriction fragments were separated on ethidium bromide-stained 3% and 2% agarose gels, respectively.

Plant and animal mtDNA sequences were obtained from GenBank (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD). With only two exceptions, the $tRNA^{Lys}$-ATPase8-ATPase6 ovine and porcine sequences (our unpublished data, available upon request) sequence comparisons and analyses were carried out with reference to the database of GenBank.

RESULTS

Bovine-specific mtDNA amplification. The mtDNA sequence encoding for the $tRNA^{Lys}$ and the subunit 8 and subunit 6 of the ATP synthase complex was chosen as the target sequence. Such a region was preferred to other mtDNA-coding sequences because both ATPase8 and ATPase6 exhibit a relatively high degree of variation among vertebrates. Furthermore, such a $tRNA^{Lys}$-ATPase8-ATPase6 gene configuration is not found in mitochondrial genomes of

TABLE 1. *Oligonucleotides tested to amplify the bovine-specific tRNA^{Lys}-ATPase8 mitochondrial sequence*

Primer[a]	Oligonucleotide
L7933	5′-CCAAACAACCCTTATATCCTC-3′
L8014	5′-ACCCATTGTCCTTGAGTTAGT-3′
L8129	5′-GCCATATACTCTCCTTGGTGACA-3′
L8159	5′-CTAGACACGTCAACATGACTGA-3′
H8230	5′-GTGTCAGTTCTGGATTGTGA-3′
H8357	5′-GTAGGCTTGGGAATAGTACGA-3′
H8518	5′-GGTTTGTTGATCCAATAAATAG-3′

[a] Primers are identified by a letter designating either the light (L) or the heavy (H) strand and a number corresponding to the reference sequence position of the base at the 3′ end of the primer, according to Anderson et al. *(2)*.

higher plants, which also apparently lack a homolog to ATPase8.

Initially, we tested different primer pairs (Table 1) and PCR conditions in order to amplify specifically the bovine target sequence. Sizes of fragments ranged approximately between 650 and 150 bp. The best results were obtained by using primers L8129 and H8357, which amplify a 271-bp region encompassing the ATPase8 gene. On the whole, sequence comparison between L8129 and H8357 primers and homologous sequences from other vertebrates indicates a low degree of homology (Table 2), particularly when considering the nonmammalian species. In any case, species specificity of the bovine L8129/H8357 amplification primarily resides in the occurrence of several primer-template mismatches at the 3′-terminal of the H8357 oligonucleotide when using mtDNA of other vertebrates as templates. Such a primer pair was utilized in an optimized PCR setting whose stringency was promoted by high annealing temperature and low primer concentration.

The bovine-specific L8129/H8357 PCR amplification is shown in Figure 1. The specificity of the oligonucleotides was evaluated by testing genomic DNAs from vertebrates whose offals are commonly included in animal feeds, i.e.,

TABLE 2. *Sequence comparison between primers L8129 and H8357 (coding strand), utilized to amplify specifically a bovine mtDNA region encompassing the ATPase8 gene, and homologous sequences from other vertebrates, including species whose offals are commonly included in animal feeds*

Primer or homolog	Nucleotide sequence																								Reference[a]
																		tRNA^{Lys} (3′)			ATPase8 (5′)				
																		↓			↓				
L8129	G	C	C	A	T	A	T	A	C	T	C	–[b]	T	C	C	T	T	G	G	T	G	A	C	A	1
Sheep	·[c]	·	A	T	G	·	·	·	·	·	·	–	·	·	·	·	·	·	A	·	·	·	·	·	2
Goat	·	·	A	T	A	·	·	·	·	·	·	–	·	·	·	·	·	·	A	·	·	·	T	·	3
Pig	·	·	·	T	A	·	A	T	·	·	·	–	C	·	·	·	C	A	A	·	·	G	T	·	2
Horse	·	T	T	C	A	·	C	C	·	C	·	–	·	·	·	C	·	A	·	·	·	·	T	·	4
Rabbit	T	A	A	·	·	·	G	T	·	·	·	–	·	·	·	A	·	A	·	·	·	·	A	·	5
Chicken	·	G	G	·	C	·	C	C	·	·	·	C	C	·	·	·	·	A	A	·	·	·	·	·	6
Duck	·	A	A	T	·	·	·	C	·	C	·	·	·	·	·	·	·	A	A	·	·	G	·	·	7
Carp	A	T	T	C	C	C	G	·	·	C	A	–	C	·	T	C	·	A	·	·	·	·	A	·	8
Rainbow trout	·	·	·	C	C	C	A	·	·	C	A	–	C	·	·	C	·	A	·	·	·	·	·	·	9
Atlantic salmon	·	T	·	C	C	C	A	·	·	C	A	–	C	·	·	C	·	A	·	·	·	·	·	·	10
Atlantic cod	A	·	T	C	C	C	A	·	·	C	A	–	C	·	·	·	·	A	A	·	·	·	A	A	11
ATPase6 (+67 bp) ↓																									
H8357 (coding strand)	T	C	G	T	A	C	T	A	T	T	C	C	C	A	A	G	C	C	T	A	C				1
Goat	·	T	A	·	T	T	·	·	·	·	T	·	·	T	·	·	·	T	·	·	·				3
Pig	·	T	A	·	T	A	·	·	·	·	·	·	·	·	·	·	·	T	·	·	A				12
Horse	·	·	A	·	C	A	·	·	·	·	T	·	·	C	·	·	·	A	·	C	·				4
Chicken	C	A	C	·	C	·	·	T	C	·	T	·	·	·	G	C	·	·	·	C	·				6
Duck	C	·	C	·	G	·	·	T	C	·	T	·	·	·	G	C	·	·	·	·	T				7
Carp	·	·	T	A	G	G	A	·	·	·	·	·	T	·	·	T	A	T	C	G	·				8
Rainbow trout[d]	C	·	T	A	G	G	·	·	·	C	·	·	A	C	T	T	A	T	C	G	·				9
Atlantic salmon[e]	C	·	T	A	G	G	C	C	A	·	·	T	·	C	T	·	·	·	G	C	A				10
Atlantic cod	C	·	T	C	G	G	A	·	·	·	·	·	·	·	T	A	A	T	·	T	T				11

[a] References (indicated as GenBank accession number): 1, J01394; 2, our unpublished data; 3, X65975; 4, X79547; 5, X64107; 6, X52392; 7, L22476 L08260; 8, X61010; 9, L29771; 10, U12145; 11, X17659; 12, M26139.

[b] Dashes indicate gaps introduced to improve alignment.

[c] Nucleotide homologies are indicated by dots.

[d] Masu salmon and Amago salmon: same sequence, except C → T at position 76. Biwa salmon: same sequence, except A → G and C → T at positions 70 and 76, respectively.

[e] Brown trout: same sequence, except A → C, G → T, and C → G at positions 70, 85, and 86, respectively.

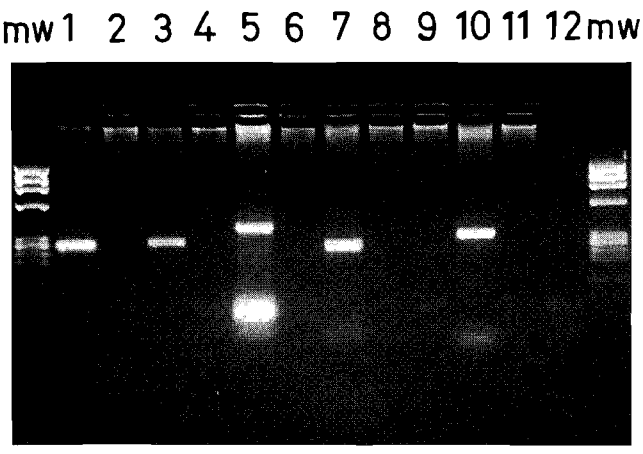

FIGURE 1. *Bovine-specific PCR amplification of the 271-bp region flanking the ATPase8 mitochondrial gene, by using the L8129 and H8357 primer pair. Lane 1: bovine genomic DNA template. Specificity of oligonucleotides was evaluated by testing genomic DNAs from sheep (lane 2), swine (lane 4), horse (lane 6), rabbit (lane 8), chicken (lane 9), and turkey (lane 11) as templates. Homologous sequences from sheep (lane 3), swine (lane 5), horse (lane 7), and chicken (lane 10), amplified by using species-specific primer pairs, as positive controls. Lane 12: PCR negative control.* Hae*III digested ΦX174 DNA was used as molecular weight (mw) marker; fragments: 1,353, 1,073, 872, 603, 310, 281/271, 234, 194, 113, and 72 base pairs.*

sheep, swine, horse, rabbit, chicken, and turkey as templates. A PCR product of the expected size was obtained from bovine genomic DNA, while no homologous product was observed by testing the other specimens. Direct sequencing confirmed the amplification of the target $tRNA^{Lys}$-ATPase8-ATPase6 sequence (data not shown).

Bovine-specific mtDNA detection from animal feeds. Twenty microliters of single-round PCR reactions which included DNA extracts from reference feedstuff samples containing 2, 1, 0.5, 0.25, and 0.125% MBM (lanes 1 to 5), together with 20 μl of PCR reactions including DNA extracts from a reference feedstuff lacking bovine-derived MBM, a pure FM sample, and both extraction and PCR negative controls (lanes 6 to 9) are shown in Figure 2. Amplification products of expected size were obtained from all reference samples containing bovine-derived MBM, giving evidence of the high efficiency of the extraction procedure and the sensitivity of the PCR. Specificity of the L8129 and H8357 primer pair is supported by the absence of homologous PCR product in both samples lacking bovine-derived MBM. The absence of amplified product from the extraction and PCR mock samples testifies that no cross- and/or carryover-contamination occurred. Priming specificity was also confirmed by a second round of amplifications containing 5 μl of first-round PCR reactions which did not result in detectable homologous products (data not shown). Repetitions of both extraction and amplification procedures were performed; in all cases results were confirmed.

A reference feedstuff set was further subjected in our laboratory to additional heating at 133°C for 33 min by autoclaving. As is apparent from Figure 2, the method was able to detect the bovine target sequence by a single-round PCR from these samples (lanes 11 to 15) also, proving that,

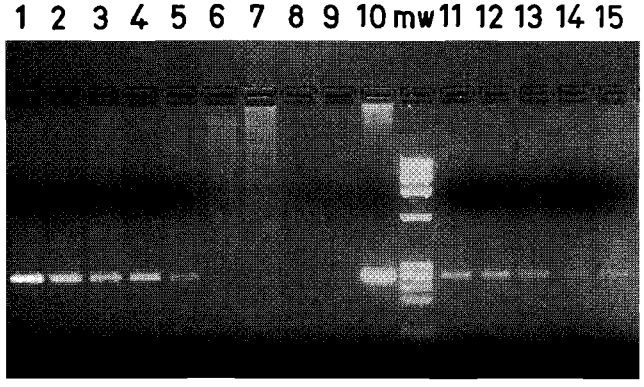

FIGURE 2. *Single-round PCR amplification of bovine mtDNA from reference feedstuffs containing bovine-derived MBM, by using the L8129 and H8357 primer pair. Lanes 1 to 5: reference samples containing 2, 1, 0.5, 0.25, and 0.125% MBM, respectively; lanes 6 to 9: sample lacking MBM, pure FM sample, and extraction and PCR mock samples, negative controls; lane 10: bovine genomic DNA sample, positive PCR control; lanes 11 to 15: single-round PCR products from the same reference feedstuffs as set of lanes 1 to 5 after additional heating at 133°C for 31 min, by autoclaving.* Hae*III digested ΦX174 DNA was used as molecular weight (mw) marker; fragments: 1,353, 1,073, 872, 603, 310, 281/271, 234, 194, 113, and 72 base pairs.*

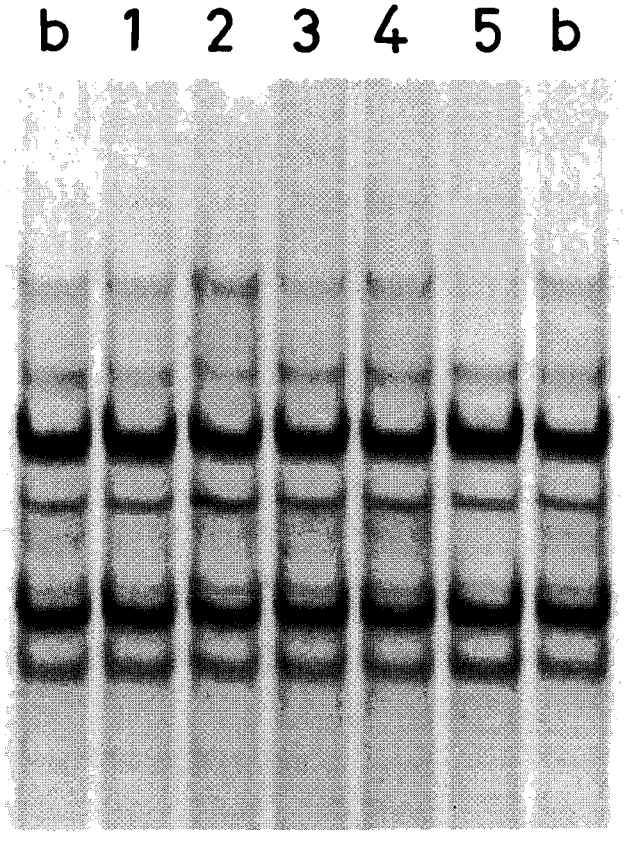

FIGURE 3. *Single-strand conformational analysis (SSCA) profile comparison between L8129/H8357 PCR products obtained from bovine genomic DNA and DNA extracts from reference feedstuffs containing bovine-derived MBM. DNA extracts from reference feedstuffs containing 2, 1, 0.5, 0.25, and 0.125% MBM (lanes 1 to 5, respectively); bovine genomic DNA (b). Samples were loaded on a 5% acrylamide (ratio 29:1) gel containing 0.5% glycerol.*

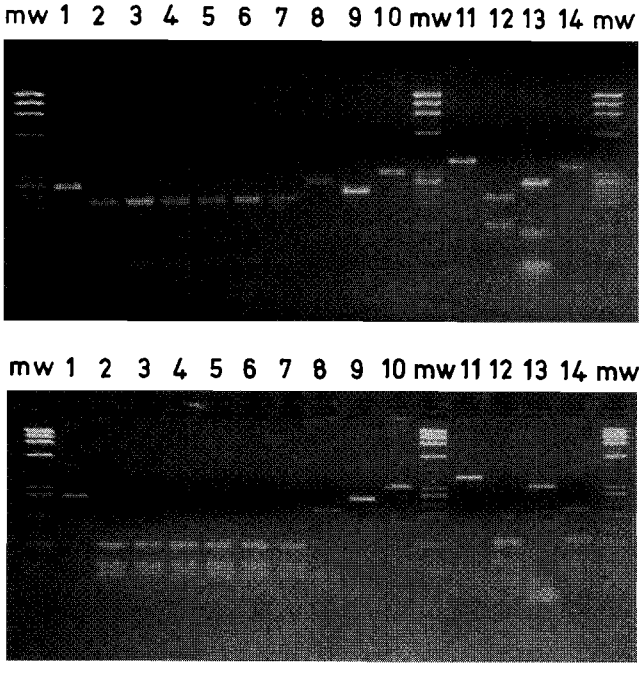

FIGURE 4. Dpn*II (above) and* Ssp*I (below) restriction profiles of the bovine and reference feedstuffs L8129/H8357 PCR products, compared with profiles of homologous sequences from other vertebrates. Lane 1: undigested bovine PCR product; lane 2: digested bovine PCR product; lanes 3 to 7: digested PCR products from reference feedstuffs containing respectively 2, 1, 0.5, 0.25, and 0.125% MBM; lane 8: digested ovine PCR product; lane 9: digested equine PCR product; lane 10: digested poultry PCR product. Lanes 11 and 12: undigested and digested bovine L8014/H8357 PCR products; lanes 13 and 14: digested porcine and ovine PCR products homologous to the bovine L8014/H8357 sequence.* Dpn*II and* Ssp*I restriction sites in the porcine PCR product are located upstream to the ATPase8 coding sequence.* Dpn*II and* Ssp*I fragments were electrophoresed on 2 and 3% agarose gels, respectively.* Hae*III digested ΦX174 DNA was used as molecular weight (mw) marker; fragments: 1,353, 1,073, 872, 603, 310, 281/271, 234, 194, 113 and 72 base pairs.*

although there was a lower number of copies, the prolonged heat treatment did not appear to considerably affect the efficiency of the assay.

Analyses of the PCR products. Second-round PCR products from 2% and 0.125% MBM-containing reference feedstuff samples were purified and sequenced on both strands. Sequences unanbiguously fit the published bovine sequence (data not shown). Identity of the PCR products from reference feedstuffs to the bovine target sequence was also evaluated by means of single-strand conformational analysis (Figure 3). SSCA has proved to be a useful tool for species identification, permitting the detection of single base changes in short fragments due to the mobility differences of single-stranded DNA molecules. Detection efficiency of even single base changes has been estimated as higher than 90% when considering sequences of 100 to 300 bp in length *(11).* SSCA patterns of PCR products from reference feedstuffs and bovine genomic DNA appeared identical, supporting identity between sequences.

Neither sequencing nor SSCA are convenient techniques in routine control assays, being time-consuming, expensive, and labor-intensive. As both *Dpn*II and *Ssp*I endonuclease digestions yield distinctive restriction profiles of the bovine L8129/H8357 PCR product, their restriction patterns could be used routinely to support the bovine origin of the amplified fragments. *Dpn*II enzyme cuts the bovine sequence into two fragments of 57 and 214 bp (Figure 4, above). We found this restriction profile to be monomorphic in a sample of 55 unrelated cattle from two European breeds (Charolais and Friesian). A single *Dpn*II site also characterizes the equine homologous sequence, but it would result in a clearly distinguishable restriction pattern (248 and 23 bp) when electrophoresed on a 3% agarose gel. Differently, as is observed in most vertebrates, homologous sequences from sheep, goat, swine, and chicken do not have any *Dpn*II restriction site. We did not find any *Dpn*II restriction site in 40 and 42 unrelated ovine and porcine samples, respectively. As regards the *Ssp*I digestion pattern, two sites characterize the bovine target sequence (Figure 4, below). Digestion leads to three fragments of 69, 84, and 118 bp. Such an expected pattern was observed in all the 55 bovine samples we tested. To our knowledge, no similar electrophoretic profile is found in homologous sequences among vertebrates. Accordingly, a single *Ssp*I site was observed in all the ovine samples (fragments of 69 and 202 bp in length), and no site was found in the porcine samples. As is apparent from Figure 4, both *Dpn*II and *Ssp*I digestions of the PCR products from reference feedstuffs resulted in the expected bovine-specific restriction patterns.

DISCUSSION

The bovine spongiform encephalopathy outbreak in Great Britain arose from feeding cattle supplemental rations containing scrapie-infected sheep tissues not adequately treated to inactivate the infectious agent, and its rapid spread also resulted from the use of feedstuffs enriched with BSE-infected bovine offals *(28).* Even if we are far from a satisfactory knowledge of the biological identity and properties of the BSE agent, two facts are currently accepted: firstly, the BSE agent is particularly resistant to several inactivation procedures *(25);* secondly, TSEs affect humans and several other mammals *(21, 24).* For this reason, in view of a possible spread of BSE and bovine-to-human BSE transmission, the use of ruminant-derived proteins in ruminant feeds has been prohibited in both the European countries and the United States. At present, a critical point concerning the observance of such a ban is represented by the absence of really effective controls. Immunoassays and bone microscopic identification, proposed as control methods to test for the presence of ruminant or mammalian proteins in animal feeds, do not appear to be effective tools, and simple, sensitive, species-specific and rapid diagnostic methods are still in demand.

Our approach consisted in the use of bovine-specific mitochondrial DNA sequences as marker for the presence of bovine-derived materials in feedstuffs. Three striking features made mtDNA the molecule of choice for our study: its high number of copies per cell *(17, 22),* its quite high mutation rate *(6),* and the difference in its gene arrangement between plants and animals *(5, 10, 16, 23, 29).* Coding sequences were preferred to noncoding sequences, i.e., the

D-loop region, in order to cut down intraspecies genetic heterogeneity. More precisely, we considered a sequence 271 bp in length encoding the 3′ portion of $tRNA^{Lys}$ and the whole subunit 8 and the amino-terminal portion of subunit 6 of the ATP synthase complex *(2)*. This region shows a relatively low degree of conservation among vertebrates, and even among Bovidae a sensible degree of variation of the ATPase8 nucleotide sequence is apparent. This region was preferred also because of its peculiar structural organization in vertebrates. More precisely, the $tRNA^{Lys}$ and ATPase8 coding sequences are nearly always separated by a single nucleotide, and the ATPase8 and ATPase6 genes overlap by 10 to 46 nucleotides. Such a $tRNA^{Lys}$-ATPase8-ATPase6 gene configuration, which is about 900 bp long, is not found in the mitochondrial genomes of higher plants, in which the $tRNA^{Lys}$ and ATPase6 genes are scattered or at any rate are not located so close together. Furthermore, no protein homologous to ATPase8 seems to be encoded by plant mtDNA *(26)*.

A simple and efficient method was utilized to recover template DNA from feedstuffs by using silica particles, which are known to bind nucleic acids in presence of chaotropic agents. This silica-based method allowed us to detect the presence of bovine mtDNA in reference feedstuffs containing less than 0.125% bovine-derived meat and bone meals. It does not appear to be considerably affected by the prolonged heat denaturation of the rendering process, and its sensitivity proved to be high also when reference samples were further subjected to additional heating at 133°C for 33 min by autoclaving. By using this method we have been able to detect the presence of bovine mitochondrial DNA in several lots of commercial feeds. Finally, the coupling of *Dpn*II and *Ssp*I endonuclease digestion of the unpurified PCR products provides a rapid tool to further confirm the bovine origin of the amplified sequence.

This is the first report of a molecular approach to test for the presence of bovine-derived material in ruminant feeds. In our opinion, this method could be used successfully as routine control assay, being highly sensitive, reproducible, rapid, simple, and not expensive. Our hope is that the present results might stimulate researchers toward a molecular approach to the improvement of tests that are still necessary for a more rigorous enforcement and monitoring of manufactured feeds.

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