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Development of a PCR Assay for the Detection of Animal Tissues in Ruminant Feeds

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ABSTRACT

The European Community ban on use of meat and bone meal in ruminant feed, as a consequence of the spread of bovine spongiform encephalopathy in Europe, has prompted a number of investigations about the possibility of detecting animal tissues in feedstuff. In this paper, a study on vertebrate primers, designed in the 16S rRNA gene of mitochondrial DNA, is described. These primers were able to amplify fragments that contained between 234 and 265 bp. The fragments were specific for bovine, porcine, goat, sheep, horse, rabbit, chicken, trout, and European pilchard and were confirmed by sequence analysis amplicons. The primers were used in a PCR assay applied to five samples of meat and blood meals of different species and subjected to severe rendering treatments (134.4 to 141.9°C and 3.03 to 4.03 bar for 24 min). The presence of vertebrate tissues was detected in all samples. The assay proved to be rapid and sensitive (detection limit 0.0625%). It can be used as a routine method to detect animal-derived ingredients in animal feedstuff.

As a consequence of the occurrence of cases of bovine spongiform encephalopathy (BSE), the use of animal-derived meals in the manufacture of feedstuff has been banned in the European Community. In fact, European Community decisions 2000/766 *(5)* and 2002/248 *(6)* establish that "Member States shall prohibit the feeding of: a) proteins derived from animals to ruminants; b) processed animal protein to farmed animals which are kept, fattened or bred for the production of food." In other countries, such as the United States, different regulations have been established in this field.

The detection of animal tissues in feedstuff is therefore an issue of great significance for the implementation of measures against the spread of BSE.

For this purpose, a microscopic method for the detection of bone fragments has been recognized as the "official" method in the European national plans against BSE. However, this method is time consuming and its reliability mainly depends on the professional skill of the microscopist.

The need for alternative analytical approaches has prompted numerous studies. The application of biomolecular techniques, which tend to be more sensitive and applicable even to heat-processed products, has gained increasing interest.

Several studies have dealt with the application of PCR for the detection of bovine tissue in animal feedstuff *(10, 13, 15, 16)*. More recently, real-time PCR has enabled the quantification of bovine tissue *(11)*. In other research *(12)*, a species-specific PCR has been developed to identify ovine, porcine, and avian tissues in meat and bone meal.

In this article, a PCR based on primers able to amplify some vertebrate DNA was developed and applied to different animal-derived feedstuff.

MATERIALS AND METHODS

Samples. Samples of 3 g of whole raw meat and of autoclave-treated meat (121°C for 15 min) from different species of surface and aquatic vertebrates were analyzed. Rodent tissues were also considered because these animals can accidentally enter the feedstuff during manufacturing, storage, or both. In addition, 3 g of some vegetable materials, always contained in feedstuff, as well as some animal materials not prohibited by the European Union law, such as milk whey powder and lard, were analyzed.

Bacteria and mold cultures and insects were tested to detect possible cross-reactions with these environmental contaminants. To verify the applicability of the technique in the feedstuff analysis, the assay also was carried out on commercial animal meals obtained from rendering industrial plants and vegetable feedstuff. All the samples considered are detailed in Table 1.

Finally, to evaluate the test sensitivity, fish meal was diluted in vegetable meal according to the legend of Figure 6, and DNA was extracted from each dilution. All the samples were accurately mixed in a vortex.

DNA extraction. The Dneasy tissue kit (Qiagen, Hilden, Germany), with minor modifications for the application to complex products, was used for the extraction from all samples, except bacteria. The modifications consisted of an increase of the sample amount from 25 to 400 mg and a decrease of the final elution volume to 100 μl. The Extman Evolution Formula (Link-Biotech, Biotechnology and Research Company, Monza, Italy), specifically developed for feedstuff analysis, was also applied. This kit is based on the use of "Twin Rex Man" resin.

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TABLE 1. *Descriptions of samples used in the PCR assay*

Sample	*n*	Species
Raw meat	9	Bovine (*Bos taurus*)
		Goat (*Capra hircus*)
		Sheep (*Ovis aries*)
		Porcine (*Sus scrofa*)
		Horse (*Equus caballus*)
		Rabbit (*Oryctolagus cuniculus*)
		Chicken (*Gallus gallus*)
		Pilchard (*Sardina pilchardus*)
		Trout (*Oncorhynchus mykiss*)
Autoclaved meat	9	Bovine (*Bos taurus*)
		Goat (*Capra hircus*)
		Sheep (*Ovis aries*)
		Pork (*Sus scrofa*)
		Horse (*Equus caballus*)
		Rabbit (*Oryctolagus cuniculus*)
		Chicken (*Gallus gallus*)
		Pilchard (*Sardina pilchardus*)
		Trout (*Oncorhynchus mykiss*)
Rodent tissue	2	*Mus musculus*
		Rattus norvegicus
Vegetables	3	Maize (*Zea mais*) (2)[a]
		Soy (*Glycine max*) (1)
Lard	1	Porcine
Milk whey powder	1	Bovine
Bacteria	10	*Salmonella anatum*
		Rhodococcus equi
		Escherichia coli
		E. coli O157:H7
		Pseudomonas aeruginosa
		Campylobacter jejuni
		Bacillus cereus
		Legionella pneumophila
		Listeria monocytogenes
		Legionella taurinensis
Mold	10	*Penicillium* spp.
		P. aurantiogriseum
		Aspergillus flavus
		A. versicolor
		A. nidulans
		A. fumigatum
		Cladosporium cladosporioides
		Fusarium sambucinum
		Rhizopus oligosporus
		Scopulariopsis brevicaulis
Mix of insects	1	Flies (*Musca domestica*)
		Bees (*Apis mellifera*)
Pure blood meal	1	Bovine
Pure meat meal	4	Bovine (2)[a]
		Fish (1)
		Pork (1)
Vegetable feedstuff	7	

[a] Number of samples in parentheses.

For bacteria, DNA samples from 10 species were kindly provided by "Camera di Commercio" Laboratory of Turin and extracted with the PrepMan kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer's recommendations.

In every extraction, some reagent controls, as well as negative samples containing heterologous DNA (from cereals), were included. A duplicate of every extraction was performed for all samples.

Evaluation of DNA denaturation. Electrophoresis on 2.5% agarose gel was performed on DNA extracted from raw meat and on meat autoclaved at 121°C for 15 min.

Primer design. In the preliminary phases of the study, the universal primers designed by Kocher et al. *(8)* amplifying *cyt.b* of mitochondrial DNA were used. In a second phase, new primers in gene 16S rRNA of mitochondrial DNA were designed after alignment using version 1.6 of Clustal W *(7)* of the sequences found in the GenBank database of the following species: bovine, goat, sheep, pork, horse, rabbit, chicken, European pilchard, and trout.

The primers (synthesized by Roche Diagnostic, Monza, Italy) were designed in a well-conserved nucleotide sequence. Oligonucleotide sets were (sense) 2509 5′AAGACGAGAAGACCCT(A/G)TGGA(A/G)CTTTA3′ and (antisense) 2742 5′GATTGCGCTGTTATCCCTAGGGTA3′ (GenBank accession no. NC 001567).

For all materials that were expected to give a negative result with the aforementioned primer set (bacteria, molds, insects, and vegetable ingredients), specific primer sets for each group were applied according to the literature: molds *(17),* insects *(9),* soy *(18),* and maize *(14).* For bacteria the MicroSeq 500 16S rDNA bacterial sequencing kit (Applied Biosystems) was employed.

For vertebrate species (if any) that were negative with the primer set proposed in the study, amplification of *cyt.b* mitochondrial DNA was carried out (as a positive control), as described by Kocher et al. *(8).*

This was to verify that a negative result was not due to low yield or poor quality of the DNA sample.

PCR procedure. Amplifications were done with the Thermal Cycler 2400 (Applied Biosystems) on a final volume of 50 μl containing 20 mM Tris-HCl (pH 8.4); 1 unit of platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, Calif.); bovine serum albumin 1% (Roche Diagnostics GmbH, Mannheim, Germany); 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia, Uppsala, Sweden); 1.5 mM $MgCl_2$; 25 pmol of each primer; and 250 to 500 ng of DNA template. After an initial denaturation step at 94°C for 5 min, 35 cycles were programmed as follows: 94°C for 30 s, 68°C for 1.30 min, final extension at 72°C for 5 min.

PCR fragments of the expected length were purified by the Concert Rapid PCR Purification System (Gibco, New York) and cycles sequenced (both strands) using PCR-derived primers on an ABI 310 genetic analyzer (Applied Biosystems) by the dideoxy chain termination method with fluorescent dye terminators (Applied Biosystems). The sequences obtained were deposited in the GenBank database (accession no. AY236425-33).

The nucleotide sequences were submitted to BLASTn sequence similarity searching *(1)* at the National Centre for Biotechnology Information database and were aligned with the bovine, goat, sheep, pork, rabbit, horse, chicken, pilchard, and trout sequences available in the GenBank database.

Other materials, such as bacteria, molds, insects, and vegetable ingredients (soy, maize, vegetable feedstuff), were also amplified by specific procedures according to published studies: Colombo et al. *(3)* for molds, Jeyaprakash and Hoy *(9)* for insects, Wurz et al. *(18)* for soja, and Studer et al. *(14)* for maize. For bacteria, the MicroSeq 500 16S rDNA bacterial sequencing kit (Applied Biosystems) was employed.

All the amplimers were resolved on 2.5% agarose electro-

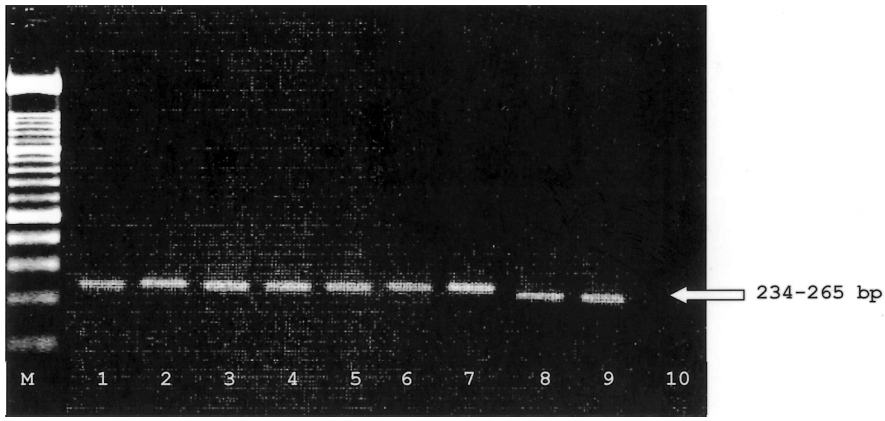

FIGURE 1. *PCR of DNA from autoclaved meat (mt16S rRNA gene). The fragments generated have a length of between 234 and 265 bp. M, 100-bp ladder; lane 1, bovine; lane 2, pork; lane 3, horse; lane 4, rabbit; lane 5, chicken; lane 6, pilchard; lane 7, trout; lane 8, goat; lane 9, sheep; lane 10, control reagent.*

phoresis carried out in Tris acetate EDTA buffer for 60 min at 120 V and stained with ethidium bromide (0.4 μg/ml for 20 min).

RESULTS

Evaluation of DNA denaturation. Electrophoresis of DNA extracted from raw meat showed a high content of fragments >500 bp, whereas DNA extracted from autoclave-treated meat showed a majority of smaller fragments (≤300 bp) (not shown).

PCR specificity. The application of primers designed by Kocher et al. *(8)* has enabled the DNA amplification in all samples from raw meat and produced the expected amplicon of 376 bp. On the other hand, the expected amplicon was detected only in a few cooked meat samples. For this reason, new primers were designed in the 16S rRNA gene of mitochondrial DNA. These new primers generated shorter amplicons, between 234 and 265 bp, in autoclave-treated samples (Fig. 1). The size of the fragment depended on the number of deletions in each vertebrate species.

Every sequence obtained in the assay was analyzed with the BLASTn sequence similarity search *(1)* of the National Center for Biotechnology Information database. A high homology rate (98 to 100%) for the same animal species was observed (Table 2).

The results for rodent DNA were controversial. A 239-bp amplicon was produced from *Mus musculus* DNA and none from *Rattus norvegicus* (Fig. 2, lanes 7 and 8).

The DNA samples of bacteria, molds, insects, soy, maize, and other vegetable materials gave no amplicons using the proposed PCR assay. These negative samples were afterwards submitted to PCR with primers specific for each group, resulting in all positive (Figs. 2 and 3). A positive result was observed also for milk whey powder and lard (Fig. 3, lanes 5 and 6).

Application to commercial meals. The products tested came from rendering plants and had been subjected to a very severe rendering treatment (134.4 to 141.9°C and 3.03 to 4.03 bar for 24 min). In vegetable feedstuff, no amplification was obtained, unlike that observed with specific primers (Fig. 4); the test detected the presence of vertebrate tissues in all samples of animal meal, suggesting that the fragment size was suitable for the analysis of degraded DNA (Fig. 5).

All the results of the specificity tests are outlined in Table 3.

PCR sensitivity. The sensitivity of the test, determined by means of different dilutions of known amounts of a fish

TABLE 2. *Sequencing results*

Sequences deposited	GenBank sequences	Homology rate (%, BLASTn)
Bos taurus AY236425	J01394	99
Sus scrofa AY236432	AF304203	100
Equus caballus AY236427	X79547	98
Oryctolagus cuniculus AY236428	AJ001588	100
Gallus gallus AY236430	X52392	100
Sardina pilchardus AY236433	AB032554[a]	92
Oncorhyncus mykiss AY236431	AF125509	99
Capra hircus AY236426	M55541	99
Ovis aries AY236429	AF010406	100

[a] *Sardinops melanostictus.*

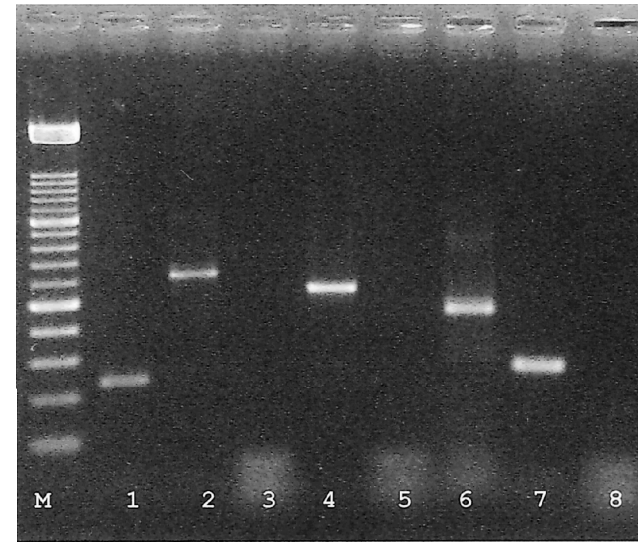

FIGURE 2. *PCR of DNA from molds, bacteria, insects, and rodents. M, 100-bp ladder; lanes 1* and 2,** molds; lanes 3* and 4,** bacteria; lanes 5* and 6,** insects; lane 7,** Mus musculus*; lanes 8* and 9,*** Rattus norvegicus. ** Tested with proposed primers; ** tested with specific primers.*

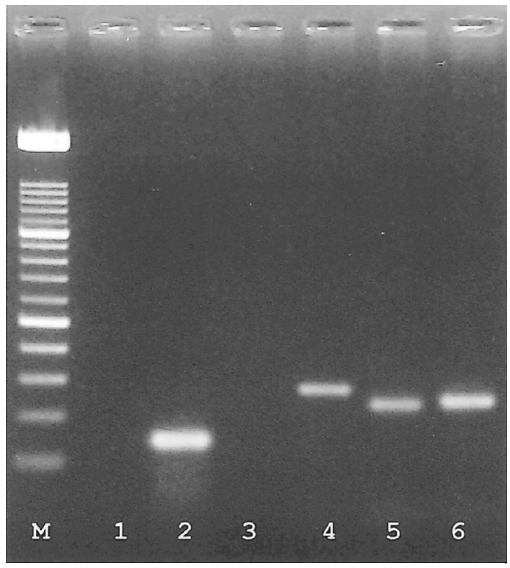

FIGURE 3. *PCR of DNA from vegetable and animal materials. M, 100-bp ladder; lanes 1* and 2,** soy; lanes 3* and 4,** maize; lane 5,* milk whey powder; lane 6,* lard. * Tested with proposed primers; ** tested with specific primers.*

meal in a vegetable meal, gave satisfactory results. In both procedures, the detection limit was 0.0625% (Fig. 6).

DISCUSSION

The aim of this study was to design primers that can be used for detection of prohibited animal materials in feedstuff.

After the European Community ban, any finding of meat and bone meals in feedstuff could be attributed either to a fraudulent addition or to a simple accidental contamination.

In the case of accidental contamination, however, the amount of "banned" products probably would be low; therefore, the method of detection would have to be highly sensitive and the analyzed sample very representative. The latter point posed significant problems both of sampling and of DNA extraction. As for sampling, the preliminary tests were made on 400 mg of sample using Dneasy Tissue kit (Qiagen). That amount proved to be unsuitable for detection of low amounts of animal materials. That is probably why the European Community directive 98/88 *(4)* on guidelines

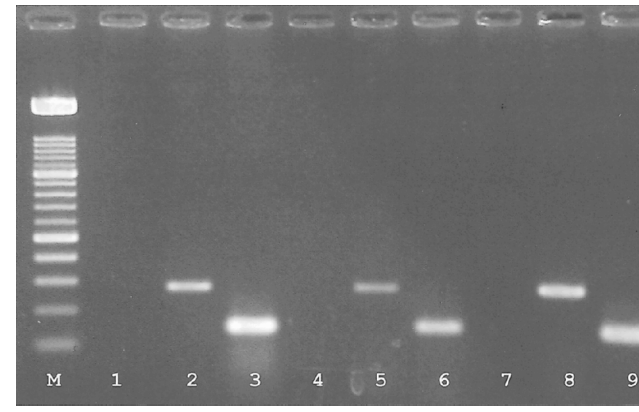

FIGURE 5. *PCR of DNA from meat and blood meals (mt16S rRNA gene). M, 100-bp ladder; lanes 1 and 2, meat meals (bovine); lane 3, blood meal (bovine); lane 4, meat meal (pork); lane 5, meat meal (fish); lane 6, positive control; lane 7, control reagent.*

for the microscopic identification of animal materials in feed, indicates an amount of 10 g of sample as adequate for the detection of bone fragments. Alternatively, we evaluated the Extman Evolution Formula (Link-Biotech, Biotechnology and Research Company), which is the first kit specifically devised for feedstuff and which is based on 5 g of material for the analysis. However, this kit has given DNA recoveries much smaller than the Dneasy Tissue kit (Qiagen). This does not appear to be a problem where animal meal consists entirely of animal-derived materials. However, a low recovery of DNA could impair the assay in feed meals where cereal meals predominate and in which only a minimal percentage of animal tissue is present. For this reason, it is advisable to use the Dneasy Tissue kit (Qiagen), possibly making two or more extractions from the same sample.

The conducted assay proved to be sensitive, with a detection limit of 0.0625% of animal DNA. This high sensitivity, on the other hand, could represent a drawback because it can be difficult to discriminate between an accidental cross-contamination subsequent to the processing of animal and cereal products in the same plant and an intentional addition of banned materials.

This drawback could be overcome by means of a quantitative PCR; Lahiff et al. *(11)* recently developed a real-

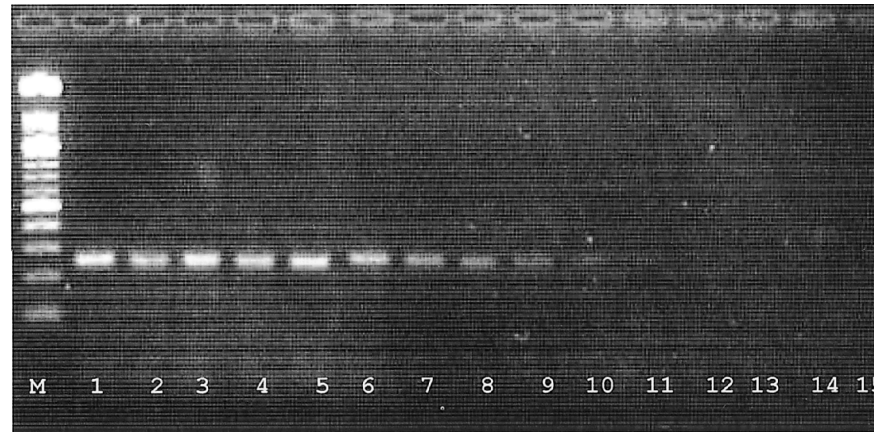

FIGURE 4. *PCR of DNA from vegetable feedstuff. M, 100-bp ladder; lanes 1,* 2,** and 3,*** vegetable feedstuff; lanes 4,* 5,** and 6,*** vegetable feedstuff; lanes 7,* 8,** and 9,*** vegetable feedstuff. * Tested with proposed primers; ** tested with specific primers for maize; *** tested with specific primers for soy.*

TABLE 3. *Results of PCR assays*

		Primer targets						
	Samples	Vertebrate[a]	Vertebrate[b]	Insects[c]	Maize[d]	Soy[e]	Molds[f]	Bacteria[g]
Species target	Raw meat ($n = 9$)	+	+					
	Autoclaved meat ($n = 9$)	+	+/−[h]					
Contaminants	Molds ($n = 10$)	−					+	
	Bacteria ($n = 10$)	−						+
	Rodents ($n = 2$)							
	Mus musculus	+	+					
	Rattus norvegicus	−	+					
	Insects	−		+				
Admitted animal ingredients	Lard	+						
	Milk whey powder	+						
Vegetable ingredients	Maize	−			+	−		
	Soy	−			−	+		
	Vegetable feedstuff	−			+	+		
Animal meal	Blood	+						
	Meat	+						

[a] Primers proposed by the authors.
[b] Kocher et al. *(8).*
[c] Jeyaprakash and Hoy *(9).*
[d] Studer et al. *(14).*
[e] Wurz et al. *(18).*
[f] White et al. *(17).*
[g] MicroSeq 500 16S rDNA bacterial sequencing kit.
[h] Inconsistent results because of the excessive length of the fragment.

time PCR able to quantify the presence of bovine material in six industrial samples that previously tested positive for the presence of bovine material with a conventional PCR assay.

Another crucial point in this kind of analysis is represented by heat degradation of DNA subsequent to the autoclaving specified by the European Community Law for thermal processing of animal ingredients. This degradation has caused some problems in PCR applications, mainly when the fragments to be amplified were small.

To avoid these difficulties, primers able to amplify a fragment of about 270 bp were designed after having noticed that it was possible to optimize the amplification only with fragments below 300 bp. Similar observations were made by Colgan et al. *(2),* who employed species-specific primers between 250 and 300 bp, and by Tartaglia et al. *(15),* who in a similar investigation designed primers of 271 bp for the identification of bovine tissue.

Alignment of the sequences obtained in this assay, along with sequences available in GenBank, revealed a high degree of homology for all the tested species, except the European pilchard, for which the similarity did not exceed 92%. A possible explanation for this could be the difference in species; in fact, the reference GenBank species was *Sardinops melanostictus,* whereas *Sardina pilchardus* was used.

The PCR on rodent tissues was performed because the presence of such tissues as environmental contaminants of cereal meals during storage is possible, although hardly likely.

The failure to detect *Rattus norvegicus* DNA in feedstuff was justified after examination of GenBank sequence alignment of all species. In fact, it was noticed that, although the homology for *Mus musculus* primer binding sites was 98%, for *Rattus norvegicus* it was 94%. In the latter species, two deletions were also observed in the antisense primer binding sites.

Although these findings contrast with vertebrate taxonomy on the basis of phylogenetic properties, the primer design strategy was carried out, taking into account the main vertebrate species that are usually processed in industrial rendering plants. Thus, these species represent the target range of the proposed assay.

The detection of some rodent tissues in vegetable meals could represent a shortcoming of this PCR technique because it could be misinterpreted as a fraudulent contamination. On the other hand, the microscopic method cannot discriminate among the different mammals, and it is not suitable to detect consistently tissues other than bone.

Because the primer set is able to amplify a wider range of vertebrate species, special attention should be used during all steps, avoiding human contamination. For example, during the validation of the PCR test, DNA samples from several mold species obtained from an independent laboratory gave amplification with length consistent with the vertebrate range, leading one to suppose a specific amplification: sequence analysis of such PCR products revealed, without a doubt, the human mt16S rRNA (BLASTn 100%).

As expected, milk whey powder and lard were easily detected by PCR. Milk whey and lard are permitted in animal feeding (Decision E.C.766/2000) *(5),* provided their presence is indicated on the label. However, even in such

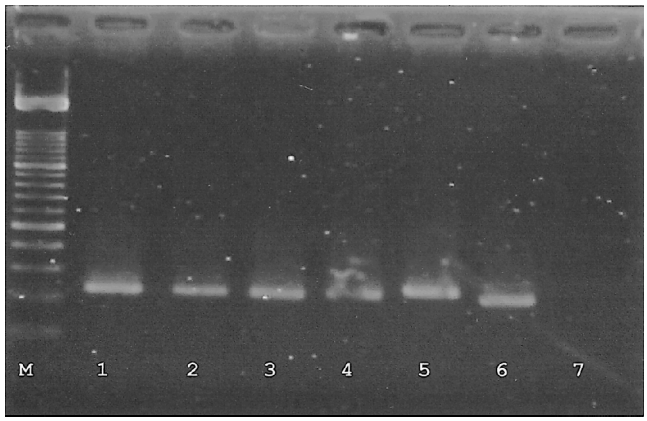

FIGURE 6. *Evaluation of assay sensitivity: scalar dilution of a fish meal in a vegetable meal. M, 100-bp ladder; lane 1, 50%; lane 2, 20%; lane 3, 10%; lane 4, 5%; lane 5, 3.3%; lane 6, 2%; lane 7, 1%; lane 8, 0.5%; lane 9, 0.25%; lane 10, 0.125%; lane 11, 0.0625%; lane 12, 0.03125%; lane 13, 0.025%; lane 14, 0.02%; lane 15, control reagent.*

a case, the presence of milk whey powder could mask the fraudulent addition of banned animal products. On the other hand, the microscopic method, which is based on the identification of bone fragments, can only detect the presence of vertebrate tissues, but fails in detecting other ingredients such as blood meal, which is equally prohibited in ruminant nutrition.

An ideal method for the indisputable identification of animal products in feedstuff does not exist as yet; therefore, it seems advisable to couple PCR to the microscopic method, as indicated in the European Community Directive 98/88 *(4)* ". . . taking into consideration the scientific and technological progress, it is advisable to associate other analytical methods to microscopic examination."

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