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Detection of Ruminant Meat and Bone Meals in Animal Feed by Real-Time Polymerase Chain Reaction: Result of an Interlaboratory Study

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The commercialization of animal feeds infected by prions proved to be the main cause of transmission of bovine spongiform encephalopathy (BSE). Therefore, feed bans were enforced, initially for ruminant feeds, and later for all feeds for farmed animals. The development and validation of analytical methods for the species-specific detection of animal proteins in animal feed has been indicated in the TSE (Transmissible Spongiform Encephalopathies) Roadmap (European Commission. *The TSE (Transmissible Spongiform Encephalopathy) roadmap.* URL: http://europa.eu.int/comm/food/food/biosafety/bse/roadmap_en.pdf, 2005) as the main condition for lifting the extended feed ban. Methods based on polymerase chain reaction (PCR) seem to be a promising solution for this aim. The main objective of this study was to determine the applicability of four different real-time PCR methods, developed by three National expert laboratories from the European Union (EU), for the detection and identification of cattle or ruminant species in typical compound feeds, fortified with meat and bone meals (MBM) from different animal species at different concentration levels. The MBM samples utilized in this study have been treated using the sterilization condition mandatory within the European Union (steam pressure sterilization at 133 °C, 3 bar, and 20 min), which is an additional challenge to the PCR methods evaluated in this study. The results indicate that the three labs applying their PCR methods were able to detect 0.1% of cattle MBM, either alone or in mixtures with different materials such as fishmeal, which demonstrates the improvement made by this technique, especially when compared with results from former interlaboratory studies.

KEYWORDS: Ruminants; beef; feed; animal meals; MBM; mitochondrial/chromosomal DNA; real-time PCR

INTRODUCTION

The spread of bovine spongiform encephalopathy (BSE) and its relation to the consumption of contaminated animal feeds led to the ban within the European Union on the use of mammalian processed animal proteins (PAPs), including meat and bone meal (MBM), as an ingredient in feed for ruminants (EC Regulation 999/2001) (*2*). The animal byproducts (ABPs) Regulation EC 1774/2002 (*3*) prohibits feeding farmed animals with proteins from the same species, because scientific advice suggested that this practice presented a risk of spreading various diseases. The lack of methods allowing species-specific identification led to the introduction of a ban of PAPs for *all* farmed animals (extended feed ban) by amending Regulation 999/2001 (*2*), through Commission Regulation 1234/2003 (*4*).

The major condition for possible changes of the extended feed ban is the improvement and validation of analytical methods to control the presence and species identification of processed animal proteins in feedingstuffs. Various methods are applied to the analysis of feed samples for the presence of banned PAPs (*5*), but at the moment classical microscopy is the only official method within the EU to detect the presence of constituents of animal origin. The analysis has two objectives: (i) the detection of MBM irrespective of the origin and (ii) the detection of MBM from terrestrial animals in the presence of fishmeal. The Commission Directive (*6*) describing the protocol of classical microscopy also allows for applying

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alternative methods to gain more information about the origin of the found PAPs, however only after the official microscopic method has been applied on the samples. Furthermore, the Commission Directive states that, with this method, very low amounts of MBM (<0.1%) can be detected in animal feed. In addition, some improvements of this technique, such as the combination with near-infrared analysis, have been reported to detect down to 0.05% of MBM in feed (*7*). However, the actual limit of detection could be different depending on various factors (e.g., the bone fraction of the MBM or the presence of fishmeal). In fact, a proper detection of 0.1% MBM in the presence of 5% of fishmeal might be possible (*8*), whereas other interlaboratory studies (*9*, *10*) revealed a significant number of false negative results when utilizing the EU official method to detect 0.1% MBM in the presence of 5% of fishmeal. Nevertheless, the limit of detection of 0.1% is set as a benchmark against which the suitability of other methods such as polymerase chain reaction (PCR) is tested. Another limitation of classical microscopy is the fact that this method does not allow for a species-specific determination of PAPs.

Alternative analytical methods have been developed for detecting animal materials in feeds. These methods are mainly based on the analysis of their protein and DNA contents. Immunoassay and PCR have been pointed out since they allow species-specific detection. But there are significant limitations that make this task difficult, mainly the need to detect traces of animal material and the denaturation and degradation of proteins and DNA due to the rendering process. In a previous intercomparison study carried out in 2003, both immunoassays and PCR showed very poor results (*9*) especially when applying PCR, where a high number of false positive and negative results were reported. Another study performed in 2004 (*10*) for the detection of PAPs in feeds by immunoassays revealed an improved performance profile of the immunoassays tested, though there were still significant differences among the tests. Differences were found with respect to the selected animal targets and—linked to it—the tissue specificity, the taxonomic level of detection and the sensitivity.

Methods based on PCR, where well-defined DNA targets are determined to detect the presence of PAPs at various taxonomic levels, seem a promising solution for the detection of animal tissue presence and animal species identification. In recent years, great improvements have been made on PCR techniques. Among methods based on PCR and usually applied for detection of animal material in feeds, three main groups can be differentiated depending on the specificity, which are (i) those allowing species-specific detection, usually of the most common farm animals such as cattle, sheep, pig or chicken; (ii) those allowing the detection of a group of species (e.g., ruminants, mammals); and (iii) those allowing the detection of any animal DNA present in samples. One of the major challenges to PCR methods regarding the detection of PAPs at trace level are the severe sterilization conditions that need to be applied within the European Union, consisting of steam pressure treatment at 133 °C, 3 bar for 20 min (*3*). Concerning the DNA targets usually chosen for this type of sample (usually highly degraded) sequences that can be found in high copy number are preferred, including mitochondrial DNA (mtDNA) or repetitive sequences in genomes like mammalian-wide interspersed (MIR), satellite DNA (*11*) or short and long interspersed nucleotide elements (SINEs and LINEs) (*12*, *13*).

Concerning mtDNA, this target has been reported to be a powerful tool for the identification of different animal species in feeds (*14*−*16*) and has also been applied for ruminant species detection in the same matrix (*17*−*22*). The selection of mtDNA is advantageous because (i) its presence in multiple copies per cell (as many as 2500 copies in a postmitotic tissue such as skeletal muscle) increases the probability of achieving a positive result, even in the case of samples undergoing intense DNA fragmentation due to severe processing conditions (*23*), and (ii) its large variability compared with nuclear sequences, which undergo a less rapid evolution, facilitating authenticity studies (*24*).

As another approach, repetitive sequences in genomes that are also present in high copy numbers, such as about 120 000 copies of mammalian-wide interspersed repeats (MIR), or 10^5 copies of short interspersed nucleotide elements (SINEs) (*12*) per mammalian genome, make this kind of sequence also very interesting for the detection of DNA targets from highly processed samples (*11*, *12*). However it is necessary to determine the real potential of these methods when applied to samples with special difficulties such as feeds, since although a large number of laboratories have developed PCR tests, only few of them have reached the minimal standards in terms of required sensitivity and specificity to be considered as a potential official method.

This study was designed for the evaluation of the species-specific detection of cattle or ruminant DNA by three different laboratories each of them using their own primers and probes. The study was composed of two parts, namely, the organization of the study which was performed by the IRMM, and the analysis of the samples by the participating laboratories. Both parts were strictly separated. This means that none of the participating laboratories were involved in establishing the experimental design of the study or in the preparation of test samples. The procedure allows for an independent assessment of the suitability of the four method fitness for the intended purpose. The performance profile of the methods was characterized in terms of (i) the sensitivity, indicating the capability of the test to correctly classify samples containing MBM as positive, and (ii) the specificity, indicating the capability of the test to correctly classify blank samples as negatives. All laboratories used real-time PCR and hybridization probes. With this technique it is possible to monitor the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle. The use of real-time PCR allows the expression of the results as numerical values (Ct values), which gives more information about the process, allowing a comparison among different samples to establish definitive results. Threshold cycle (Ct) values are calculated by determining the point at which the fluorescence produced in each sample reaches the chosen threshold limit, and it is inversely related to the starting copy number of the target sequence (*25*).

The study was performed on behalf of the European Commission's Direction General Health and Consumer Protection and illustrates the current state of the art of four different real-time PCR methods. The methods, developed by three National expert laboratories, were designed for the detection of cattle or ruminant DNA in feed samples, and were selected for showing promising performance (results not published). Participating laboratories were the Walloon Agricultural Research Centre (CRA-W) in Belgium, the Netherlands Organisation for Applied Scientific Research (TNO), and the Veterinary Laboratories Agency (VLA) from the U.K.

MATERIALS AND METHODS

Description of Test Materials. Fifteen test materials were sent in blind triplicates to each of the three participating laboratories. For the preparation of these test materials, compound feeds intended for cattle,

pig, and chicken feeding were used, either alone or fortified with MBM at different concentration levels. All samples were prepared individually by adding the exact amount of MBM to each sample item to ensure that the correct percentage of animal meals was present in each of them. The blank compound feed samples were prepared in a feed mill containing typical ingredients such as soybean meal, maize, wheat, or barley.

The final composition of the test materials (MAT I to MAT XV), as shown in **Table 1**, was established taking into account the following aspects to correctly evaluate the different methods: (i) the use of different compound feeds to mimic a "real world" situation and to assess the effect of the presence of a high number of quite different ingredients in the feed on the performance of the methods, (ii) the use of heat treated MBM to assess the influence of the DNA breakage due to heat treatment on the performance of the methods, (iii) the target concentration of cattle MBM in feed at 0.1%, reflecting its presence at contaminant level, and (iv) the presence of fishmeal, feathermeal, or porcine MBM at 5% level either alone or with 0.1% cattle MBM to check the specificity and sensitivity of the methods.

The feeds were obtained from a feed mill with high quality standard which performs its own check by classical microscopy to ensure that no MBM is present. Additionally, the feeds were checked at the IRMM by immunoassays for presence of ruminant material, which was negative in all cases except for one of the cattle feeds and one of the pig feeds, which were additionally checked by near-infrared microscopy (NIRM) and by the official European method, with a negative result in both cases.

Concerning the animal meals, the pure cattle and pig MBM were obtained from a pilot plant and produced from species pure byproducts of each considered animal species (cattle, pig). The meals were treated at a temperature of 133 °C and 3 bar for 20 min as required by European legislation (*3*). Afterward the material was dried under atmospheric conditions until the moisture content was below 10%. Finally the product was pressed and ground. These materials were also checked, by PCR in this case, in order to establish the species included, and the results indicated that bovine and porcine MBM contain respectively only bovine and porcine DNA.

The fishmeal was obtained directly from a fishmeal producer, and the feathermeal was obtained from a pilot plant and produced from poultry byproducts.

Requested Information from the Participating Laboratories. The laboratories were asked to report on the detection of ruminants or bovine DNA to determine the performance of the methods used. The results had to be given as a qualitative response, so the laboratories had three options to report the results: (i) present, (ii) not present, and (iii) no results. The latter response corresponded to inconsistent results or was indicated by the laboratories when the method did not allow the detection of the indicated animal species or group of species.

As mentioned above, laboratories were asked to indicate the Ct values obtained for each sample and for each of the target parameters. Although quantitative results were not possible at the moment due to the specific characteristics of materials such as complex composition, heat treatments, and presence of different animal tissues, the Ct values were helpful to evaluate qualitative results, especially when comparing the sensitivity and specificity of the different analytical methods. In this context it is important to note that a high Ct value corresponds to a low initial concentration of the target amplicon and vice versa. Likewise it is important to mention that each laboratory selected and reported a specific cutoff Ct value, for each method, to distinguish between positive and negative samples, and these cutoffs were determined empirically by individual studies performed at the different laboratories prior to the receipt of the blinded samples. These studies were performed by each laboratory with the most common farm animal species to test their specificity, being the results positive just for the intended animal species or group of species (ruminant or cattle) in every case on the range of the cutoff value.

DNA Extraction. Because contaminated particles may not be uniformly dispersed throughout a feed, batch it is vital that appropriate sampling techniques are employed in order to generate an accurate and reproducible result. As seen in **Table 2**, each laboratory applied the

Table 1. Description of Test Materials Included in the Study and Results Reported by the Participants[a]

		MAT I		MAT II		MAT III		MAT IV		MAT V		MAT VI		MAT VII		MAT VIII		MAT IX		MAT X		MAT XI		MAT XII		MAT XIII		MAT XIV		MAT XV	
														Concentration (%)																	
cattle MBM 133 °C		–		–		–		0.1		0.1		0.1		0.5		0.5		0.5		–		–		–		–		0.1		0.1	
porcine MBM 133 °C		–		–		–		–		–		–		–		–		–		5		5		–		–		–		–	
feathermeal		–		–		–		–		–		–		–		–		–		–		–		–		5		–		5	
fishmeal		–		–		–		–		–		–		–		–		–		–		–		5		–		5		–	
compound feed		100[c]		100[p]		100[ch]		99.9[c]		99.9[p]		99.9[ch]		99.5[c]		99.5[p]		99.5[ch]		95[c]		95[p]		95[c]		95[p]		94.9[c]		94.9[p]	
samples per lab		3		3		3		3		3		3		3		3		3		3		3		3		3		3		3	
														Results[b]																	
target	**lab**	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**	**P**	**N**
cattle	CRA-W	0	3	**3**	0	0	3	3	0	3	0	3	0	3	0	3	0	**3**	0	**3**	0	0	3	**3**	0	3	0	3	0	3	0
	TNO	0	3	**3**	0	0	3	3	0	3	0	3	0	3	0	3	0	**3**	0	**3**	0	0	3	**3**	0	3	0	3	0	3	0
	VLA	0	3	0	3	0	3	3	0	3	0	3	0	3	0	3	0	2	**1**	0	3	0	3	0	3	3	0	3	0	3	0
ruminant	TNO	0	3	**3**	0	0	3	3	0	3	0	3	0	3	0	3	0	3	0	**3**	0	0	3	**3**	0	3	0	3	0	3	0

[a] Key: [c]Compound feed intended for cattle. [p]Compound feed intended for pig. [ch]Compound feed intended for chicken. [b] Incorrect results in bold, italics, and underlined; P, positive; N, negative.

Table 2. Protocols Used by Participating Laboratories

	CRA-W	TNO	VLA
	DNA Extraction/Purification Protocol		
test portion size	100 mg × 2	1 g	40 g
method used	magnetic beads	guanidine HCl/magnetic beads	Chelex resin
	Amplification Protocol		
DNA target	mitochondrial DNA	highly repetitive genomic sequences	mitochondrial DNA
species detected/amplicon size	cattle/68 bp	ruminants/83 bp cattle/142 bp	cattle/108 bp
cutoff limit[a]	40 cycles	35 cycles for ruminant 40 cycles for cattle	35 cycles

[a] Limit to distinguish between positive and negative samples.

procedure for DNA extraction and purification that it considered suitable for the purpose.

The procedure applied by CRA-W consisted of a first step in which samples were ground on a ZM200 mill (Retsch GmbH & Co., Haan, Germany) to obtain a powder of particles with a diameter <500 μm before extraction. After this step DNA was extracted and purified in duplicate from a 100 mg test portion with the commercial kit "Wizard Magnetic DNA Purification System for Food" (Promega Corporation, Madison, WI) according to the supplier's instructions and using the King Fisher Magnetic Particle Processor (Thermo Labsystems, Helsinki, Finland) as a semiautomatic device for performing these extractions. Final DNA extract was recovered in 300 μL.

DNA extraction was performed by TNO after the milling of the samples to a fine and homogeneous mixture; 1 g was taken from this mixture and mixed with TNE buffer containing guanidine HCl (5 M) and Proteinase K. DNA purification was performed using the Wizard DNA cleanup system (Promega) following the protocol from the supplier. As reference samples, pig feed spiked with cattle meal heat treated at 133 °C at 0.1, 0.5, and 5% was used (CCL, NL).

VLA developed a method for extracting DNA from the feed sample starting from a larger test sample (40 g) in order to ensure the detection of target DNA in samples, and using a Chelex resin DNA extraction/purification protocol. With this method, the test portion is soaked in a phosphate buffer to release material from the sampled pellets. The soaked sample is preprocessed in order to release the DNA into the buffer. A subaliquot of the buffer is treated with Chelex, vortexed for 20 s, and then centrifuged for 10 min. The liquid is then removed and is ready for testing.

Real-Time PCR Analysis. For the real-time PCR analysis each laboratory used its own primers and probes and its own protocols as seen in **Table 2**.

For the method developed by CRA-W (*21*), each PCR reaction was performed on 5 μL of undiluted extract and on a 10-fold dilution to check for possible inhibition, in duplicates for each test portion. Real-time PCR was performed with an ABI5700 thermocycler (Applied Biosystems, Foster City, CA) and in a total volume of 35 μL containing 5 μL of template DNA (3-fold or 30-fold diluted Promega extract to check for inhibition), 17.5 μL of qPCR Mastermix (Eurogentec, Belgium), 0.75 μL of each primer at 5 μM (Eurogentec), 2.5 μL of the appropriate TaqMan probe at 5 μM (Eurogentec), and 8.5 μL of PCR-grade water (ICN Biomedicals, Belgium). The Ct given as result is a mean of the Ct for each of the four (two test portions and two PCRs per test portion) undiluted extracts, as there appeared to be no significant inhibition.

For both methods developed by TNO, real-time PCR was performed using a Gene Amp 5700 or an ABI Prism 7700 instrument (Applied Biosystems), and 10 μL of each sample or reference was used, in duplicates, for each amplification reaction together with 15 μL of PCR mix containing 12.07 μL of TaqMan universal master mix (Applied Biosystems), 1.1 μL of each primer at 10 μM, and 0.73 μL of the probe at 5 μM. Both methods were previously tested with test materials obtained from well-defined raw materials from single species such as avian, ovine, porcine, and bovine, treated under controlled rendering conditions at different temperatures, and also with different commercial samples including fish meal, milk powder, or feather meal. As a result the cutoff Ct value was established for each method.

For the method developed by VLA, real-time PCR was performed and detected on a 7900HT sequence detector (Applied Biosystems) in a total volume of 25 μL containing 12.45 μL of TaqMan Mastermix (Applied Biosystems), 0.07 μL of forward primer, and 0.08 μL of reverse primer at 0.3 μM and 0.13 μL of probe labeled with fluorescent reporter dye 6-carboxyfluorescein (FAM) at 0.1 μM. In addition an internal positive control (IPC) is added to each test well by adding 1 μL of IPC template at a 15000-fold dilution, 0.01 μL of IPC forward primer and 0.04 μL of IPC reverse primer at 0.03 μM and 0.19 μL of IPC probe VIC labeled 0.15 μM. This involves the amplification of a region of the ampicillin resistant gene commonly found in commercial nucleic acid vectors but not naturally found in animal or plant genomes. It is a noncompetitive exogenous control and is included in the assays to detect false negative results that may arise as a result of inhibitory factors present in the samples. The internal positive control template, primers, and probes are added to the TaqMan master mix to allow multiplex detection. The primers were used at a limiting concentration to prevent IPC product utilizing the PCR reagents to the detriment of the mtDNA target amplification efficiency. Each assay is run with four nontemplate controls, six target species positive controls (0.2% 133 °C MBM in a negative feed), two lots of each nontarget species, and two negative feed controls. Cutoff values are generated on an assay by assay basis using positive control data. This method has been in-house validated and is accredited under the ISO 17025:2005 quality standard at the VLA laboratory.

RESULTS

As shown in **Table 1**, all tests confirmed 100% sensitivity for samples containing 0.1% cattle MBM either alone (MAT IV, V, and VI), where 27 out of 27 samples were correctly identified, or in mixture with fish or feathermeals (MAT XIV and XV, respectively), where 18 out of 18 were also correctly identified.

Concerning blank test samples, those test samples consisting of either cattle or chicken compound feeds were correctly classified as negative results, while the analyses of pig feeds gave false positive results for CRA-W and TNO (MAT II, XI, and XIII). Given the fact that all blank feed samples were prepared at a high technical standard and considering the negative results on the blank samples using either immunoassays or classical and NIR microscopy, the presence of MBM in the blank samples at trace level was considered as very unlikely. Therefore typical ingredients of compound feed were evaluated against the possibility of introducing target ruminant DNA in the feed that could lead to a false positive result. In a former study (*26*) it was shown that ruminant fat (tallow) could be identified by PCR due to DNA traces present in the residual insoluble impurities (RIIs) of the fat. The identification of tallow by PCR was even possible when the tallow did not contain more than 0.15% RIIs and when the tallow was mixed to porcine fat (lard) at a concentration of 2%. Since six out of eight pig and chicken compound feeds contained animal fat, whereas none

of the cattle feed did, false positive results on the blank materials could be explained by the presence of animal fat.

In order to clarify these results, an additional set of samples was prepared and sent to the participating laboratories to clearly understand the applicability of the PCR technique and to figure out possible problems related to these materials. This set of samples consisted of a sample of cattle feed blank mixed with bovine fat from the rendering industry in triplicate. This bovine fat was from a rendering process (*26*) with a maximum content of RIIs of 0.15% and was added to the cattlefeed at a 4% level, which is the typical level in which animal fats are used as ingredients in feeds. CRA-W was able to detect bovine DNA on the 3 cattle feeds containing bovine fat at a 4% level, while TNO gave 2 out of 3 positive results. On the other side, VLA was not able to detect bovine DNA in any of the samples, which was in concordance with the results from the study, pointing out that this method is less sensitive, but on the other side it had no problem to detect 0.1% cattle MBM, indicating that the sensitivity would be as required for the intended purpose.

Concerning false negatives, as seen in **Table 1**, just one sample was incorrectly classified as negative by VLA. This sample belonged to MAT IX, and in this case no cattle DNA was detected although the cattle MBM content was quite high (0.5%). The fact that the other two samples of the same materials were correctly classified as positives and all samples corresponding to MAT IV, V, VI, XIV, and XV with a cattle MBM content of 0.1% were correctly classified as positives, shows that this result is not representative of the performance of the method.

As stated before, in this study, laboratories reported Ct values which can be a useful indicator of the level of contamination with animal DNA, helping to discriminate among false and real positive values. For CRA-W, the Ct values corresponding to the false positive result for cattle detection were higher than those corresponding to samples containing 0.1% cattle MBM. As an example the values reported for MAT II, MAT XIII, and MAT X, all false positives, can be compared with the values for MAT V, MAT XV, and MAT IV, their equivalents but containing 0.1% cattle MBM, where a difference of at least 3 cycles can be observed between them (**Table 3**). This indicates that these false positives have probably a cattle DNA content below the one of samples containing 0.1% cattle MBM. The same trend was observed for TNO for the ruminant target, which uses a cutoff value of 35 for the Ct value. Again, the Ct values of the *blank* samples were higher than those of the samples with 0.1% MBM, indicating that the concentration of the DNA traces detected in the blank samples are below the one of the samples containing 0.1% cattle MBM.

In the case of cattle DNA detection for TNO, although this difference is not so clear at first sight, if Ct values from samples with 0.1% cattle MBM are compared with those from 5% porcine MBM both in cattle feed, an analogous difference is also observed.

On the other side, samples with pig feed as compound feed have closer Ct values to the equivalents containing 0.1% cattle MBM, probably due to the fact that most DNA targets are issued from the MBM.

Table 3. Reported Ct Values for False Positive Samples and for Samples Containing 0.1% Cattle MBM (Target Level)

	false positives		0.1% cattle MBM	
lab	test material	Ct value	test material	Ct value
CRA-W[a]	MAT II	37	MAT V	34
		40		34
		38		34
	MAT XIII	37	MAT XV	34
		38		33
		37		31
	MAT X	39	MAT IV	33
		40		35
		40		35
TNO[b]	MAT II	30	MAT V	27
		30		27
		31		25
	MAT XIII	31	MAT XV	26
		32		26
		31		26
	MAT X	33	MAT IV	26
		32		25
		33		26
TNO[a]	MAT II	37	MAT V	35
		38		36
		38		35
	MAT XIII	38	MAT XV	35
		36		35
		36		35
	MAT X	39	MAT IV	36
		39		35
		39		36

[a] Cattle target. [b] Ruminant target.

DISCUSSION

As stated above, all laboratories correctly classified all samples containing 0.1% cattle MBM. This indicates that the methods have been remarkably improved with respect to those in the intercomparison study conducted in 2003 (*9*) in which many laboratories had problems to detect cattle MBM at this concentration level. One of the factors that may have contributed to this improvement is, as shown in **Table 2**, that the sizes of amplicons are smaller than in the previous study, ranging from 68 bp to 142 bp. Amplicon size has been pointed out as one of the critical parameters that affect amplification efficiency (*21*, *27*). Theoretically in each amplification cycle the template molecule should be doubled, in case of optimal amplification efficiency. For the detection of animal DNA in feed samples, it is important that the target DNA is not too long, since DNA is highly degraded during the rendering process (*15*, *28*), leading to false negative results corresponding to a lack of sufficient sensitivity.

The use of real-time PCR compared to end-time PCR by all the groups is also an important factor that has contributed to the improvement of the applicability of PCR methods to the determination of highly processed material in animal feed. This is mainly due to the fact that real-time PCR allows for utilizing very small amplicons whereas the need for gel visualization (*27*) when applying end-time PCR makes difficult the selection of very small targets. Also the use of fluorogenic probes increases specificity of assays, since with this approach a positive identification requires the effective binding of a specific probe in addition to the binding of the PCR primers (*29*). Therefore, the use of real-time PCR combined with the use of small amplicons of about 100 bp was an important prerequisite for the high sensitivity of the tests evaluated in this study, especially when considering the severe heat treatment of the MBMs.

The location of target DNA is also among the factors that can affect amplification rates. The two methods developed by CRA-W and VLA used mtDNA as target molecule, while the two methods developed by TNO were based on highly repetitive genomic sequences which also contributed to the mentioned improvement.

Another remarkable fact is that, although the three laboratories used different sample size, ranging from 100 mg to 40 g, this did not affect the sensitivity of the method. Also, the use of positive controls, as in one of the methods, is especially interesting in these kinds of complex samples to detect the presence of any inhibitory effect that might lead to false negative results. In this case, it is important to optimize the reaction to avoid a reduction on the efficiency leading to less sensitivity.

It is important to highlight, as well, that none of the laboratories reported false negative results for samples with 0.1% of cattle MBM in the presence of 5% of fishmeal (MAT XIV), which is an interesting improvement with respect to the microscopic method for which this kind of material has shown to be problematic in recent collaborative and validation studies (*10*). Also no false positive results were reported on feed samples containing exclusively 5% of fishmeal (MAT XII).

Concerning the results from the blank feed samples it can be concluded that the presence of animal fat such as tallow from the rendering industry as an ingredient in feeds might lead to false positive results, when checking for the presence of banned meat and bone meal. Here we need to point out that a positive PCR response due to the presence of authorized feed ingredients such as tallow which contains traces of the target DNA is *only* a false positive result from the *legal point of view*, because the positive result can be understood as the proof of the presence of *banned* MBM. However, such a result is *not* a false positive result from a *scientific perspective*, since the target DNA has been introduced into the feed via tallow. In fact, the detection of DNA traces even in purified tallow indicates rather the high sensitivity of at least three of the PCR methods evaluated in this study. The presence in compound feeds of other authorized ingredients—such as blood meal or milk that may be sources of animal DNA— may also give positive results by this technique.

A careful study of Ct values of the *false positives* indicates that reducing the cutoff value—established for these three methods to distinguish between positive and negative samples—could be considered to avoid the *false positive* results due to the presence of DNA traces. By having this cutoff level at a high number of cycles, on one hand, the methods are able to detect cattle or ruminant DNA at trace level leading to false positive results from the legal point of view as mentioned above, and, on the other hand, the method may also lose specificity. Even with the use of specific primers and probes, after a high number of cycles it is possible to obtain a residual fluorescent signal (*21*, *29*) which could lead to a false positive result; this fact also demonstrates the importance of careful setting of the cutoff level. For reconsidering the cutoff level there are some factors that have to be taken into consideration, such as the efficiency of DNA extraction by the different methods and DNA content in samples, that can be affected by many factors such as treatments or storage which need further study, especially of the most typical ingredients used in feeds, since these factors might lead to an increase on the Ct value due to the degradation of target DNA. At the moment the combined application of the various methods needs to be considered when evaluating the positive response of a PCR analysis. In fact, negative results of other techniques to detect MBM (e.g., immunoassay or classical microscopy) might be considered as an indication for the presence of DNA origin from such authorized feed ingredients.

This study is particularly important because it shows, for the first time within the EU, that four independently developed PCR tests scored very well when applied to real world samples consisting of compound feed and heat treated MBM. This represents a huge improvement in the performance of this technique compared to the previous study from 2003 (*9*), mainly due to the combination of factors mentioned above. All of this makes real-time PCR a promising technique to be used as complementary to the official microscopy method overcoming some of its limitations which will significantly improve the overall control of the MBM ban in the EU.

ABBREVIATIONS USED

MBM, meat and bone meals; BSE, bovine spongiform encephalopathy; PAPs, processed animal proteins; mtDNA, mitochondrial DNA; Ct, cycle threshold; IPC, internal positive control.

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