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Effective PCR detection of animal species in highly processed animal byproducts and compound feeds

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Abstract In this paper we present a polymerase chain reaction (PCR)-based method for detecting meat and bone meal (MBM) in compound feedingstuffs. By choosing adequate DNA targets from an appropriate localisation in the genome, the real-time PCR method developed here proved to be robust to severe heat treatment of the MBM, showing high sensitivity in the detection of MBM. The method developed here permits the specific detection of processed pig and cattle materials treated at 134 °C in various feed matrices down to a limit of detection of about 0.1%. This technique has also been successfully applied to well-characterised MBM samples heated to as high as 141 °C, as well as to various blind feed samples with very low MBM contents. Finally, the method also passed several official European ring trials.

Keywords Feed · Meat and bone meal · (MBM) · BSE · Polymerase chain reaction · Real time PCR · Mitochondrial DNA · Species identification · Pig · Cattle

Introduction

The outbreak of bovine spongiform encephalopathy (BSE) caused the European Union to take several decisions in order to avoid the transmission of its causal agent through the food chain. At present, processed animal proteins

(PAPs), including meat and bone meal, are banished from use as feed ingredients for all farmed animals. Moreover, the use of PAPs is controlled within the European Union through several regulations. Regulation (EC) 999/2001 [1] prohibits explicitly the feeding of mammalian PAPs to ruminants. A temporary MBM ban for all farmed animals was established in 2001 and changed into a permanent MBM ban by amending the annex of Regulation (EC) 999/2001 through Regulation (EC) 1234/2003 [2]. Secondly, the animal byproduct (ABP) regulation EC 1774/2002 [3] prohibits the feeding of animals with proteins from the same species, and established three categories of ABPs which reflect different levels of food safety, including the risk due to transmissible spongiform encephalopathy (TSE). In consequence, only material from category 3 which comprises material fit for human consumption can be used to feed farmed animals. At present, classical optical microscopy is the only official method of detecting PAPs in compound feeds or in their ingredients in the European Union [4]. This technique is very sensitive, at least when bone fragments are present, but cannot discriminate between species. Moreover, the presence of fishmeal decreases the sensitivity of the method for the detection of meat and bone meal from terrestrial animals in feed [5]. There is a tremendous need for techniques that can enable us to detect these highly processed animal byproducts more routinely throughout the feed chain, as well as to identify their origin at the species level. When specific control methods are developed and validated, the total MBM ban may be reappraised [6].

To this end, several methods are being proposed. An immunoassay-based test was developed by Ansfield et al. [7], but the sensitivity of this very laborious assay is closely related to the heat treatment of the MBM [8]. More recently, two easy-to-use dipstick tests were developed: ReVeal Ruminant (Neogen Corporation, Lansing, MI, USA) and FeedChek (Strategic Diagnostics Inc., Newark, DE, USA). The specificity of these two tests is however limited to the detection of respectively ruminant or mammalian materials [8], and appears to be highly tissue-dependent [5].

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Near-infrared spectroscopic (NIRS) techniques have also been used to detect the adulteration of fishmeal with MBM [9–12], but they also show a lack of specificity (at least up to species level). There are some other very promising methods, such as the ones combining a microscope with near-infrared spectroscopy (NIRM) [13–16] or a NIR camera [17–19] but, unlike the molecular methods already used for genetically modified organisms (GMO) detection, they require equipment not widely used in control laboratories. Moreover, up to now they have provided relatively limited discrimination of the animal species.

DNA-based methods and particularly PCR techniques could help to solve at least some of these specific problems linked to the detection of MBM. In 1998, Tartaglia et al. [20] proposed a method for the detection of bovine DNA in feed. However, the results of an interlaboratory study indicated that this protocol lacked sensitivity, which was most likely due to the severe temperature treatment of the MBM used for the preparation of the test material [21]. So far, all reported attempts to use such techniques on correctly heat-treated MBM material (steam pressure sterilization at a minimum of 133 °C for not less than 20 min at a pressure of 3 bars on particles with a maximum size of 50 mm [3]) have led to the conclusion that amplification doesn't work, as DNA degradation is too important [22] or may lead to the decay of the target DNA amplicons [23, 24]. In 2003, an intercomparison study conducted to determine the presence of PAPs including MBM from various species in animal feed showed that animal-specific determination of MBM by PCR gave poor results, with the notable exception of the method described hereafter [25]. Tests for the detection of animal tissues in feeds were also reviewed by Momcilovic and Rasooly [26] and Gizzi et al. [27].

In this article we will prove that correctly heat-treated MBM and even products drastically heat-treated (up to 141 °C) with saturated steam still contain enough (species-specific) DNA to be able to perform PCR with good sensitivity and specificity. The target limit of detection for MBM in feed was set at 0.1%, which corresponds to the level achievable by classical microscopy [4]. Moreover, we also provide evidence, obtained from successful results in official European ring trials, of the feasibility of our approach to the detection of low levels of MBM in compound feeds or in feed ingredients and of the absolute requirement to use small targets [27]. Finally, we stress the

highly process-dependent nature of the DNA degradation achieved during rendering.

Materials and methods

Description of MBM, fishmeals and feeds analysed

The value of this work becomes much clearer when one has a certain level of knowledge about the samples used (composition, effective heat treatment conditions of MBM,...). This is why the samples are described extensively here.

Five MBM samples (MBM I to MBM V, Table 1) were produced in the Protec industrial rendering plant (Orsingen, Germany), which comprised an autoclave equipped with a stirrer and subsequent process units for drying, defatting and milling the material. The procedure ensures that autoclavation takes place with saturated steam, as required by European legislation [3]. Five different batches were treated at defined temperatures containing nearly the same amount of bovine and porcine material with about 20% carcasses and 80% byproducts from slaughter houses, such as offal and bones. The intended sterilization temperatures were 138 °C (MBM I), 136 °C (MBM II), 133 °C (MBM III), 129 °C (MBM IV) and 126 °C (MBM V). It must be pointed out that the sterilisation temperature used in a rendering plant can only be adjusted to a tolerance of 1–3 °C. Therefore, the exact temperatures were measured with an internal probe and were respectively 141 °C, 138 °C, 135 °C, 130 °C and 125 °C. Processing of the five runs was performed in one day, starting with the highest temperature conditions in order to avoid cross-contamination of high temperature-treated material with that treated at lower temperatures. During each run, samples were taken through a bypass from the autoclave after the 20 minutes of sterilisation at the predefined temperatures had been completed. The post-sterilization processes (defatting and drying) performed on the samples taken were done under laboratory conditions in order to apply the same treatment to all five samples, because these steps could not have been performed in the rendering plant with material treated below the required legal temperature (after collection of material through the bypass, the remaining bulk was treated at legally correct conditions). These laboratory treatments consisted of a water-evaporating step in an oven at 140 °C until most of the water was evaporated (it should be stressed that during this step the

Table 1 Influence of MBM heat treatment on C_t value (detection with cattle probe-primer system)

Samples	Composition	Heat-treatment	C_t
MBM I	~50% cattle, ~50% pig	Steam pressure autoclaving at 141 °C, 20 min	38.2
MBM II	~50% cattle, ~50% pig	Steam pressure autoclaving at 138 °C, 20 min	36.1
MBM III	~50% cattle, ~50% pig	Steam pressure autoclaving at 135 °C, 20 min	34.3
MBM IV	~50% cattle, ~50% pig	Steam pressure autoclaving at 130 °C, 20 min	30.8
MBM V	~50% cattle, ~50% pig	Steam pressure autoclaving at 125 °C, 20 min	29.6
AUSTRALIAN MBM	100% cattle	Dry rendering above 100 °C, 30 min	18.5
AMERICAN MBM	100% cattle	Dry rendering at about 132 °C (in the heating jacket not in the sample), 15 min	19.3

temperature of the material itself never exceeded 120 °C), defatting by *n*-hexane extraction, hexane evaporation and a final reduction in particle size by blending.

In order to confirm the relevance of the assay, whichever heat treatment process was used, samples of MBM and feeds containing MBM originating from outside of the European Union were also collected and analysed.

An American MBM exclusively produced from cattle and treated according a continuous rendering process at about 132 °C (within the heating device, not in the sample) for about 15 minutes with no pressure (dry heating) was obtained thanks to Dr. Frank Klein from Neogen Corporation (Lansing, MI, USA).

With the collaboration of the AGAL (Australian Government Analytical Laboratory), analyses were realised on an Australian sample of pure ruminant MBM, a vegetal feed matrix and the same matrix spiked at 0.5% with this pure Australian ruminant MBM. The rendering process to which this Australian MBM was submitted consisted of a continuous dry rendering process using a steam jacket that was heated to 134 °C. As it is a continuous process, it is difficult to be accurate about the precise duration of the treatment. However, it is estimated that the meat material reaches at least 100 °C for about 30 minutes. There is no requirement in Australia for a particle size of <50 µm.

A set of 25 samples produced for a European Commission validation study [25] was provided by the European Joint Research Center of Ispra (JRC-IHCP). These samples were made from two different compound feeds spiked at different levels (0%, 0.1%, 0.5%, 1.1%, 2.0% in mass fractions) of MBM, produced in a rendering plant under controlled sterilisation conditions (133 °C, 3 bars, 20 minutes). The rendering process was identical to the “Protec Orsingen” process described above. The bovine and porcine portions of this MBM were set to about 50% each. The MBM contents of 15 samples covering the range from 0 to 2% was known before the analysis was conducted, whereas nine samples (H20 to H29) were analysed blind.

A set of 20 fishmeals deliberately adulterated with MBM at 3%, 6% and 9% (by mass) fractions was obtained from the Scottish Agricultural College (SAC). These samples (F 01 to F 20) are extensively described in Murray et al. [9].

The method was also tested in two intercomparison studies, in which the analyses were carried on blind samples: the first one was conducted on behalf of the European Commission DG SANCO [8]. The samples (DGS 01 to DGS 24, Table 2) included in the study consisted of a triplicate of eight test materials. All of them were made of a compound feed for bovines that contained typical feed ingredients based on a realistic ration. Mammalian MBM with almost equal portions of porcine and bovine material that had been heat treated under steam pressure at 127 °C or 134 °C for 20 minutes was included in some of them at 0.1% or 0.5% levels (mass fractions). PAPs that would possibly interfere with the detection methods were incorporated into some samples at 5% and these consisted either of poultry meal treated at 133 °C and

3 bars for 20 minutes or fishmeal. The exact formula of each material is described in the final report of the study [25] and in the corresponding publication [8]. The second sample set (I 01 to I 35, Table 3) was issued from a reproducibility study that was organised in 2005 by three Italian institutes: the “Centro di Referenza per le Encefalopatie Animali” (Torino, Italy), the “Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d’Aosta” (Torino, Italy) and the “Centro di Referenza per la Sorveglianza e il Controllo degli Alimenti per gli Animali” (Brescia, Italy). The set was composed of 35 samples prepared using a common matrix made from a commercial feed for calves. The adulterated samples included a 0.1% concentration of cattle, chicken or fish PAPs. The cattle MBM was autoclaved according to the European requirements in commercial rendering plants (Marchis, personal communication).

DNA extraction

The Promega Wizard Magnetic DNA Purification System for Food (performed with the KingFisher Magnetic Particle Processor from LabSystems) was used to extract DNA from 100 mg of the sample according to the supplier’s

Table 2 Analysis of compound feed samples (DGS 01 to DGS 24) with various amounts of MBM (for descriptions of the materials, see the main text)

Sample number	Feedingstuff animal content	Detection of cattle	Detection of pig
DGS 01	0.5% MBM*	+	+
DGS 02	Blank	–	–
DGS 03	Poultry meal	–	–
DGS 04	0.1% MBM*	+	+
DGS 05	0.1% MBM* + Fishmeal	+	+
DGS 06	0.5% MBM* + Fishmeal	+	+
DGS 07	Fishmeal	–	–
DGS 08	0.5% MBM* + Poultry meal	+	+
DGS 09	Blank	–	–
DGS 10	Poultry meal	–	–
DGS 11	0.1% MBM* + Fishmeal	–	–
DGS 12	Fishmeal	–	–
DGS 13	0.5% MBM*	+	+
DGS 14	0.1% MBM*	+	+
DGS 15	0.5% MBM* + Poultry meal	+	+
DGS 16	0.1% MBM*	+	+
DGS 17	Poultry meal	–	–
DGS 18	0.5% MBM*	+	+
DGS 19	Fishmeal	–	–
DGS 20	0.5% MBM* + Fishmeal	+	+
DGS 21	0.1% MBM* + Fishmeal	+	+
DGS 22	0.5% MBM* + Poultry meal	+	+
DGS 23	0.5% MBM* + Fishmeal	+	+
DGS 24	Blank	–	–

* The bovine and the porcine portions of the MBM were set to about 50% each

Table 3 Analysis of compound feed samples (I 01 to I 35) with various amounts of MBM (for descriptions of the materials, see the main text)

Sample number	Feedingstuff animal content	Detection of cattle	Detection of pig
I 01	Blank	–	–
I 02	0.1% mammalian MBM	+	–
I 03	Blank	–	–
I 04	0.1% Fishmeal	–	–
I 05	Blank	–	–
I 06	Blank	–	–
I 07	Blank	–	–
I 08	Blank	–	–
I 09	0.1% Fishmeal	–	–
I 10	Blank	–	–
I 11	Blank	–	–
I 12	Blank	–	–
I 13	Blank	–	–
I 14	0.1% poultry meal	–	+
I 15	Blank	–	–
I 16	0.1% poultry meal	+	–
I 17	Blank	–	–
I 18	Blank	–	–
I 19	Blank	–	–
I 20	Blank	–	–
I 21	0.1% Fishmeal	–	–
I 22	Blank	–	–
I 23	Blank	–	–
I 24	Blank	–	–
I 25	0.1% mammalian MBM	+	–
I 26	Blank	–	–
I 27	0.1% Fishmeal	–	–
I 28	0.1% mammalian MBM	+	–
I 29	Blank	–	–
I 30	Blank	–	–
I 31	Blank	–	–
I 32	Blank	–	–
I 33	0.1% poultry meal	–	+
I 34	Blank	–	–
I 35	Blank	–	–

instructions. The DNA extracted was resuspended in 300 µl of water (using this volume, the extract is considered to be diluted threefold). A classical phenol/chloroform extraction procedure [28] was also used for the extraction of DNA from 200 mg of sample. In this

extraction method, the DNA extracted is resuspended in 600 µl of water (using this volume, the extract is considered to be diluted threefold).

Real-time PCR analysis

The real-time amplification methods proposed are based on the selective amplification of a fragment of 68 bp located in the mitochondrial DNA (tRNA^{Lys}/ATP8). The *TaqMan* probe (FAM–TAMRA) technology ensures the specific detection of cattle using ATP-UN/B1 as forward primer and ATP-UN/B2 as reverse primer. In the same way, another *TaqMan* probe was designed for the specific detection of pig with ATP-UN/C1 as forward primer and ATP-UN/C2 as reverse primer. Primers and probes were designed using Primer Express version 2.0.0 (Applied Biosystems, Foster City, CA, USA). Table 4 gives the characteristics of the amplicons, the primers and probes used for the detection of cattle and pig.

Real-time PCR was performed in a total volume of 35 µl containing 5 µl of template DNA (Promega extract diluted threefold or 30-fold; phenol/chloroform extract diluted 30-fold or 300-fold), 17.5 µl of qPCR Mastermix (Eurogentec, Seraing, Belgium), 0.75 µl of each primer at 5 µM (Invitrogen, Breda, The Netherlands), 2.5 µl of the appropriate *TaqMan* probe at 5 µM (Eurogentec) and 12 µl of PCR-grade water (ICN Biomedicals, Asse-Relegem, Belgium).

The amplifications were performed and optimised on a GeneAmp 5700 real-time PCR device (Applied Biosystems). The usual real-time PCR conditions were improved upon as follows: the first stage was performed at 50 °C for 2 minutes, denaturation was performed at 95 °C for 10 minutes, and 50 cycles were performed including a melting step at 95 °C for 15 s with an annealing/elongation step at 50 °C for 60 s. All results were analysed with the GeneAmp 5700 SDS version 1.3 software (Applied Biosystems).

Results and discussion

The PCR approach developed at CRA-W is based on decisive choices: i) the selected target overlaps two genes (tRNA^{Lys}/ATP8) where the succession is typical of the animal species [20, 29]; ii) the target region is present in multicopy in the cells (mitochondrial DNA), which is

Table 4 Main characteristics of targets with primers and probes used

Species targeted	Amplicon characteristics	Oligonucleotide	Length (in bp)	Melting temperature T_m (in °C)
Cattle	Length: 68 bp T_m : 77 °C	Direct primer ATP-UN/B1	17	35
		Reverse primer ATP-UN/B2	16	38
		Probe ATP-UN/B	24	53
Pig	Length: 68 bp T_m : 77 °C	Direct primer ATP-UN/C1	17	35
		Reverse primer ATP-UN/C2	16	33
		Probe ATP-UN/C	24	55

important to achieving test good sensitivity; iii) the small length of the target (68 bp) is essential considering the possible degradation state of the DNA in drastically processed animal materials [27, 30]; and iv) hybridization probes with appropriate cycle conditions are used and these increase the specificity of the test. The primer–probe combinations described above (see the “Materials and methods” section) were designed and tested for cattle and pig, but combinations for other species (chicken, sheep and fish) are already under development at CRA-W.

Primer and probe specificity

The specificity of this species detection system was confirmed (results not shown) by testing for several plant materials (corn, wheat, barley, soybean, sunflower, oats, rapeseed, chicory) and animal species (cattle, sheep, goat, pig, horse, deer, chicken, turkey, salmon, cod, trout, sole, mackerel) with up to 50 amplification cycles.

The localisation of the target is of great importance. Indeed, some studies have shown that mitochondrial pseudogenes could exist in the genomic DNA, leading to specificity problems [31]. This would be the case for the cytochrome *b* gene. Choosing a target which overlaps two genes reduces this risk and increases the ability to distinguish between other species containing the same genes but in a different way.

Influence of MBM sample heat treatment conditions and target length on PCR results

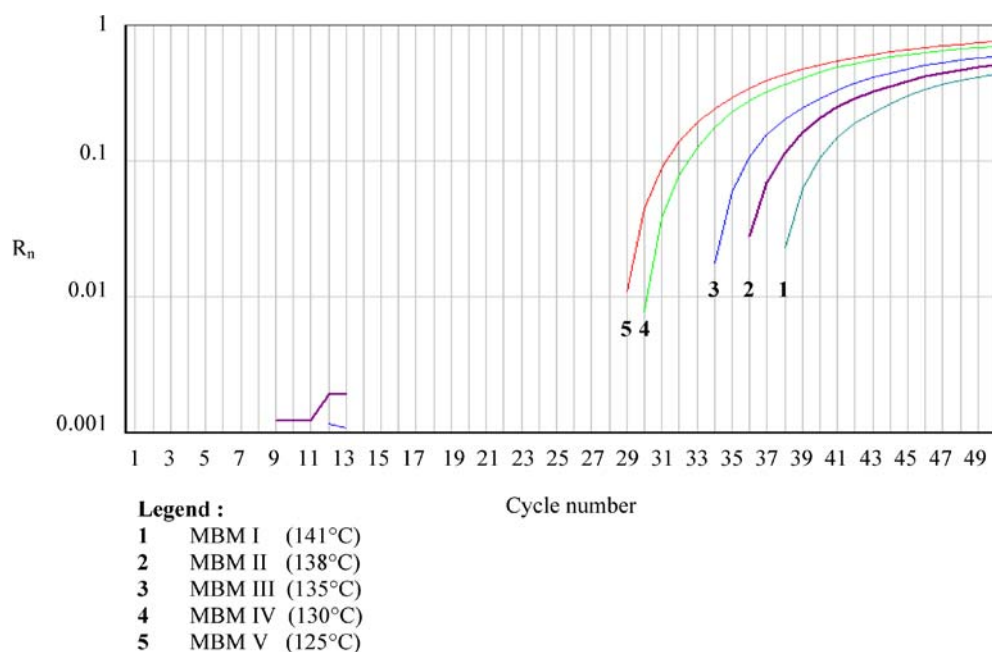
The potential of the primer–probe combination was first evaluated on DNA extracted from MBM I, II, III, IV and V. All five MBMs gave a positive response for pig and cattle,

even when treated at 141 °C (Fig. 1 shows the results for cattle). The starting point of the amplification’s exponential area clearly depends on the heat treatment endured by the sample. More intense heat treatment delays the signal (i.e., it results in higher C_t values, where C_t is defined as the number of cycles that are needed to reach a defined relative fluorescence level).

In order to demonstrate the crucial importance of the short length of the used target (68 bp) to the feasibility of detection in such samples, the reverse primer was shifted to reach fragment lengths of 113, 174, 275 and 350 bp (Fig. 2). The lengths of the different amplicons were deliberately chosen to be close to the lengths of targets previously described in the literature for the detection of MBM (108 bp [32]; 271 bp [20]; 274 bp [33]; 359 bp [34, 35]). The results obtained showed that no fragment beyond a size of 174 bp could be detected for these five MBM, although all of the targets amplified well on DNA collected from fresh meat or blood (Fig. 3a,b). Moreover, how quickly the signals arise (measured via C_t) with the three smallest targets is clearly dependent on the target size. The larger the target, the longer it takes for the signal to occur. This result is in accordance with Frezza et al. [23], who showed that amplicons of 147 bp can be used to detect the presence of bovine mtDNA in MBM samples treated according to the current European regulations.

The conditions of the sterilisation process (temperature, steam pressure, duration) have an indisputable effect on DNA damage. Therefore, PCR tests were performed on American and Australian MBM samples that were subjected to typical rendering treatments applied in their respective countries of origin. The resulting signals were compared to those obtained with MBM I to V during the same amplification run (Table 1). This clearly demonstrates that the C_t values decrease as the sterilisation conditions become less severe, i.e., as we move towards lower

Fig. 1 Detection of cattle by real-time PCR in five MBM samples treated at different temperatures (R_n =relative fluorescence units)



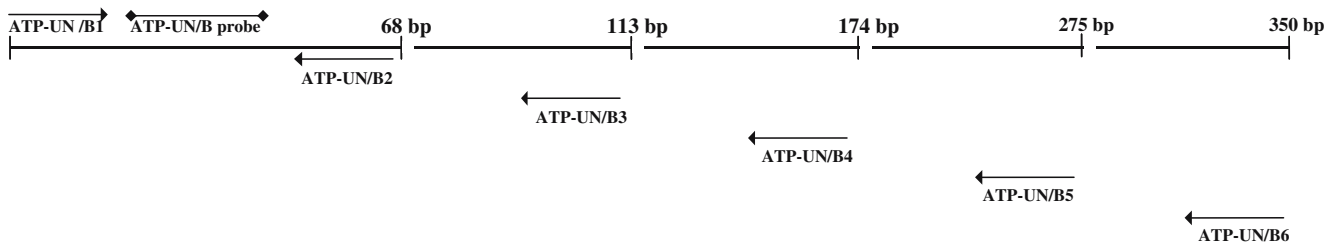
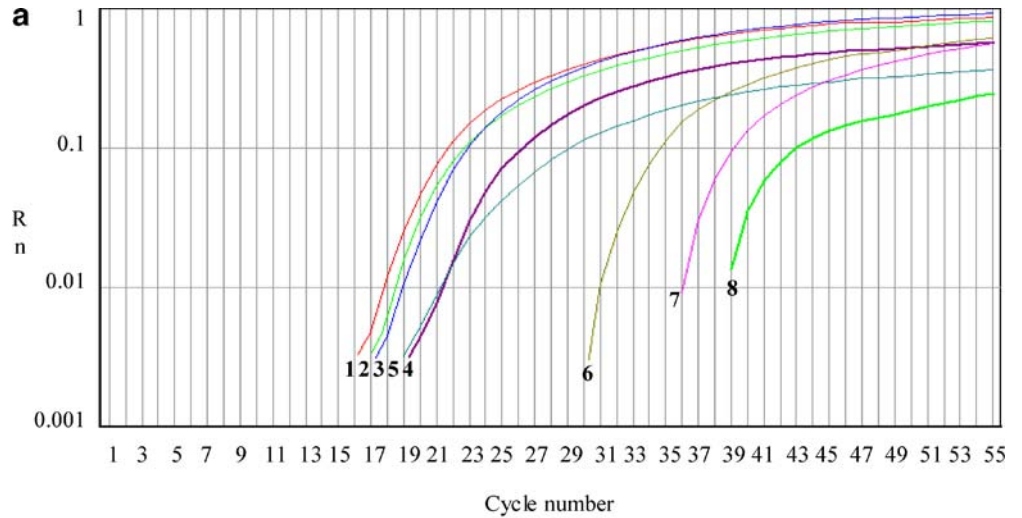


Fig. 2 Descriptions of amplicons of increasing length amplified by real-time PCR

temperature treatment carried out without steam pressure. Therefore, the signals obtained with the two non-European meals appear much earlier. Given the large range of C_t values, which vary from about 18 to 38 depending on the heat treatment, the effect of the higher content of bovine

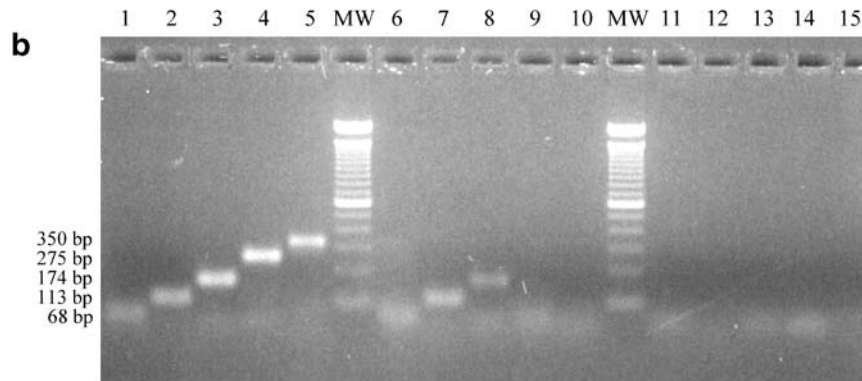
material in the American and Australian MBMs (pure cattle) compared to the other MBMs (cattle representing roughly half of the mass fraction) is considered to be negligible (it could theoretically account for a difference of

Fig. 3a–b Effect of the target length on the species-specific detection of cattle in MBM produced at different heat treatments: (a) results from real-time PCR, (b) results from endpoint detection by agarose gel electrophoresis



Legend :

1	Cattle meat	68 bp	6	MBM141°C	68 bp	ns*	no signal
2	Cattle meat	113 bp	7	MBM141°C	113 bp		
3	Cattle meat	174 bp	8	MBM141°C	174 bp		
4	Cattle meat	275 bp	ns*	MBM141°C	275 bp		
5	Cattle meat	350 bp	ns*	MBM141°C	350 bp		



Legend :

MW : molecular weight marker 100 bp

1 to 5 : cattle meat

6 to 10 : MBM 141°C

11 to 15 : negative controls

approximately one C_t unit as the number of targets is ideally increased twofold after each cycle).

Detection of MBM in fishmeal

The method developed here was also applied blind to 20 adulterated and unadulterated fishmeals (F 01 to F 20) containing MBM contents ranging from 3 to 9% (Table 5). All positive samples were clearly detected with both probes. The negative samples gave no signals, except for two samples (F 04 and F 11) which gave very late but reproducible amplification curves suggesting the presence of trace levels of animal DNA. These samples were also found to be suspicious by the near-infrared microscopy method, pointing to probable contamination during the preparation of these two samples [15].

Detection of MBM in feedingstuffs

Analysis of sample set H 20 to H 29

Next, the blind samples H 20 to H 29, 15 comparable samples with known contents in the same range as H 20 to H 29, were analysed (data not shown). For the samples H 20 to H29, the results with the pig primers/probe are given in Table 6. All samples containing 0.1% MBM were correctly identified as being positive. Since the MBM used in the trials contained 50% porcine material and the

Table 5 Detection of cattle and pig in fishmeals, some of which were spiked with MBM

Sample number	Concentration of MBM* (%)	Detection of cattle	Detection of pig
F 01	3	+	+
F 02	9	+	+
F 03	6	+	+
F 04	0	Traces	+
F 05	0	–	–
F 06	0	–	–
F 07	0	–	–
F 08	9	+	+
F 09	0	–	–
F 10	9	+	+
F 11	0	Traces	Traces
F 12	3	+	+
F 13	0	–	–
F 14	3	+	+
F 15	6	+	+
F 16	0	–	–
F 17	3	+	+
F 18	3	+	+
F 19	0	–	–
F 20	0	–	–

* The bovine and the porcine portions of the MBM were set at about 50% each

identification was performed at species level, the actual limit of detection is even lower (0.05% MBM). There was one false positive, but the other sample without MBM was correctly found to be below the detection limit of the test. On the other hand, the cattle primers/probe gave positive signals for all 25 samples, suggesting the presence of a source of bovine DNA in the feed matrix before spiking with MBM. These results were confirmed by analyses performed with the ReVeal Ruminant in Feed test (Neogen Corporation, Lansing, MI, USA), which detects the presence of ruminant muscular protein. These results provide evidence that the method utilised in this paper is a significant improvement on the PCR method [20] which was submitted to validation on the same material [25]. In this validation study [25], about 58% of all 0% animal meal samples were found to be positive and about 42% of the samples containing animal meal gave false negatives, irrespective of the concentration of animal meal in the samples. The length of the target used for this ring test (271 bp), which was too long given the heat treatment of the MBM utilised and the possible presence of another source of bovine DNA besides the MBM added to the feed, could be the reason for the higher number of false negative and false positive results.

Since all of the samples (the 15 known ones and the nine blind ones) were produced with the same MBM, a semi-quantitative approach was attempted with the pig probe. The results presented in Table 6 correspond well to the true MBM concentrations in the samples. However, even if a “quantitative calibration” was successfully achieved with this set of samples, an accurate estimation of MBM content with PCR is, in our opinion, impossible in practice. Various parameters aside from the MBM concentration, including the temperature of the heat treatment, the rendering process selected, the composition of the meat or bone meal, and possible PCR inhibition all influence the PCR signal and the C_t value. Therefore, the measured C_t value of a feed sample does not permit quantification of the MBM when these

Table 6 Analysis of compound feed samples H 20 to H 29 with various amounts of MBM (for a description of the materials, see the text)

Sample number	Concentration of MBM* (%)	Estimated % of MBM
H 20	0.5	0.1–0.5
H 21	0	0
H 22	0.1	0.1–0.5
H 23	2	1–2
H 24	1	1–2
H 25	2	1–2
H 26	0	0.1–0.5
H 27	0.1	0.1
H 28	0.5	1–2
H 29	1	0.1–0.5

* The bovine and the porcine portions of the MBM were set at about 50% each

parameters are unknown, as there is no relation between the copy numbers of the target and the weight of the MBM.

Analysis of sample set DGS 01 to DGS 24

The results for the cattle and pig targets (Table 2) were correct for 23 out of 24 samples and no false positives were obtained. Only one MBM sample, with 0.1% MBM in the presence of fishmeal, was not detected (sample DGS 11). Nevertheless, many difficulties were experienced with all of the samples containing 0.1% of MBM. In these cases, the real target concentration was in fact closer to 0.05%, as the MBM used consisted of approximately half cattle and half pig. Such a contamination level is probably below the detection limit of the method.

Analysis of sample set I 01 to I 35

The results for the cattle and pig targets are shown in Table 6. All of the samples containing 0.1% of a mammalian MBM consisting of cattle material were detected. All of the blank samples (in the sense of no addition of PAPs) were found to be negative for both cattle and pig detection. Only three samples containing chicken meal were wrongly declared to be positive: once for cattle detection and twice for pig detection.

In light of the results stated above, most of the results published in previous papers must be re-examined. First, we clearly show that PCR targets longer than 174 bp [20, 22, 36–41] can be utilised to detect fresh, cooked or canned meat but not for the detection of MBM that was steam pressure-sterilised above 133 °C. Second, it is essential to test the selected targets with well-described MBMs that undergo real-world sterilisation conditions according to European legislation. In contrast, the sample descriptions given are usually incomplete, since the exact origin and the composition [37, 38, 42] and/or the preparation of the samples are not specified. In this case, even the production process can have a wide influence on the signals. In addition, methods performed on custom autoclaved meat, even at 133 °C or higher [39, 40, 43, 44], should be treated with caution when applied to MBM analysis. Third, the limit of detection is not easy to determine. Limits of detection based on experiments with mixed DNA samples are not realistic. Indeed, the composition of the MBM, the heat treatment, matrix effects such as a PCR inhibition, and the influence of the extraction step must all be taken into account.

It should also be pointed out that other sources of cattle or pig DNA present in the sample can be detected. Indeed, the method is very sensitive and so most of the false positive results obtained probably reflected the actual presence of trace levels of bovine DNA. Furthermore, the bovine target presented here had already been successfully tested in order to determine the origin of the animal fats

[45]. PCR analyses are able to detect a contamination level as low as 5% of tallow in a matrix of other fats. PCR could even reach the 2% level, but this performance level is in fact highly dependent on the type of tallow to be analysed and the process to which it was submitted. When analysing tallow of the highest quality, “premier jus”, immunoassay and PCR techniques are not applicable since the amount of insolubles (proteins and DNA left) is too low. Blood meal and milk powder samples also give positive responses for cattle, and this fact could explain the positive signals obtained for all 25 feedingstuffs from the JRC with the cattle probe. One solution would be to undertake a specific treatment before DNA extraction, such as sedimentation, in order to eliminate other sources of DNA beyond meat and bones. Another way would be to use a multidisciplinary approach, that makes use of the power of ELISA techniques (e.g., for the detection of milk proteins) or spectroscopic methods [46] for the analysis of problematic samples. However, the real-time PCR technique remains a powerful and a highly valuable tool for the detection of various processed and heat-treated samples. Moreover, this technique could be easily applied to other species in a multiplex approach, allowing the simultaneous detection of various species in just one reaction vial.

Conclusion

Our experiments have disclosed some requirements for the application of PCR detection to heavily treated MBM samples. First, the length of the target must be minimised in order to address the DNA degradation that occurs in such samples. Using this approach, it is even possible to perform a PCR detection test on samples treated at >140 °C. Secondly, the localisation of the target is also of great importance. Choosing a target overlapping two genes in the mitochondrial DNA reduces the risk of specificity problems and increases the sensitivity of the method. Thirdly, the use of the real-time PCR technology encourages good, sensible detection by providing an historical view of the amplification by PCR. It should be stressed that the TaqMan probe again reinforces the specificity of the PCR.

The real-time PCR is as yet the only species-specific technique available, but it can also be used to detect sources of animal protein other than MBM. Used in combination with complementary techniques such as immunoassays and spectroscopic methods, it remains an important part of the approach used to determine the origins of animal proteins present in feeds.

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