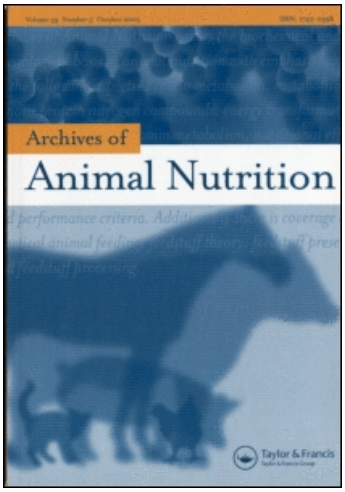

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Influences on the sensitivity of real-time PCR for the detection of bovine DNA in heat-sterilised feedstuffs

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The present study evaluated two previously developed methods for amplification of bovine mtDNA segments of 109 and 271 base pairs (bp) by real-time polymerase chain reaction (PCR). Beef samples were sterilised experimentally at different temperatures (126°C, 129°C, 132°C and 135°C). These experimentally sterilised beef samples and nine commercial meat and bone meals (MBM) were mixed to a reference plant concentrate in strengths of 50%, 10%, 5%, and 1%. The results of the following PCR showed that the Bos-109 real-time PCR assay was able to detect all the experimental beef samples with exception of the mixtures of beef heated experimentally to 135°C. In mixtures of industrial MBM bovine DNA were always found. Comparatively, the beef sterilised at 135°C and 132°C (and their respective mixtures) and the mixture containing 1% of beef sterilised at 129°C were not detectable with the PCR assay amplifying a target of 271 bp. Using this PCR mixtures of industrial MBM were only weakly detected. The low concentrated mixtures of the extremely processed MBM-1 and MBM-2 even reported negative. These results indicate that the detectability of bovine DNA is strongly influenced by the degree of the thermal treatment. Only the PCR assay amplifying relatively short fragments of a multi-copy mitochondrial target was reliable for the detection of correctly heated MBM in mixed feed.

Keywords: bovine spongiform encephalopathy; meat and bone meal; mitochondrial DNA; polymerase chain reaction

1. Introduction

As a result of the increasing occurrence of bovine spongiform encephalopathy (BSE) in cattle, measurements were taken by the European Union (EU) to control the spread of the disease. Firstly, the European Commission (1994) banned the feeding of processed animal proteins to animals reared for food production, except for the feeding of fish meal to nonruminant species. Secondly, the heat treatment of animal material (particle size of less than 50 mm) should be at $\geq$133°C for $\geq$20 min and at a pressure of $\geq$3 bar according to the European Commission (1996), in order to guarantee the denaturation of BSE agent in animal by-products. Although this is a permanent ban, the enforcement is not active, since the species-to-species ban is temporarily overruled by the extended feed ban (European Union 2003), which amends the Transmissible Spongiform Encephalopathy (TSE) regulation. These

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rules are that all animal proteins from farmed animals are prohibited from being fed back to farm animals and are due to the lack of animal-specific detection methods. After lifting (parts of) this extended ban, the species to-species ban will be actively enforced. While the technical control of the rendering plants using measures of temperature and pressure is mandatory within the EU, there is a need for a method that can be used for surveillance of the sterilised product and the detection of animal-derived substances in feedstuff to prevent the spread of BSE. Recently, a range of analytical approaches have been taken for determining the animal species in a wide array of degraded and processed substrates, mainly based on DNA or protein detection.

Polymerase chain reaction (PCR) has proven to be a versatile tool in molecular biology. Currently, real-time PCR is a powerful improvement on the basic PCR technique. Up to now several PCR assays targeting various bovine sequences have been developed for the detection of bovine tissues in composed feedstuffs. The aim of the present study was to investigate the relationship between DNA degradation, owing to the intensity of meat and bone meal (MBM) heat treatment, and the sensitivity of MBM detection in bovine feed using PCR assays. Two real-time PCR assays amplifying segments of 109 bp and 271 bp of bovine mitochondrial DNA (mtDNA) were comparatively evaluated to obtain additional information about the influence of the length of the amplicon.

2. Materials and methods

2.1. Test materials

Four meat samples (B-135; B-132; B-129; and B-126) from cattle (300 g) were cut into 50 mm cubes and sterilised separately for 20 min at 135°C and 3.3 bar, 132°C and 3.1 bar, 129°C and 2.8 bar, and 126°C and 2.6 bar pressure, respectively.

The sterilisation was in a laboratory autoclave (Varioclav, steam steriliser 400E, H+P Labortechnik GmbH, Oberschleißheim, Germany). The temperature and pressure were controlled digitally on the autoclave's control panel. The autoclaved meat samples were dried overnight in an oven at 80°C, then ground into meal. A ground plant feed mixture composed of 25% each of soya bean, corn, wheat, and pea was thoroughly mixed using Grindomix GM 200 (Retsch GmbH, Haan, Germany).

Nine industrial MBM samples (MBM-1 to MBM-9) of unknown composition were included in the test. They were obtained from different sources in Germany; MBM-1, MBM-2 and MBM-3 were obtained from a single rendering plant in the German federal state Saxony-Anhalt and were subjected to strong heat treatments (about 137°C and 3.4 bar pressure for 24 min). The other six MBM samples, obtained from different sources in Germany, were also processed according to the European Commission Decision 96/449/EC, as documented by the technical monitoring device.

For control of the efficiency of the experimental heat treatment of beef, analogous samples of pork were prepared, autoclaved and analysed with an Enzyme Linked ImmunoSorbent Assay (ELISA) using the MBM Thermal Evaluation Kit (ELISA-TEK^{TM} PORK M & BM KIT, Transia GmbH Ober-Mörlen, Germany), according to the manufacturer's instructions. All the industrial MBM samples were directly tested with the MBM Thermal Evaluation Kit to determine whether the MBM was properly heated and to compare the effect with heat treatments of beef in

the laboratory autoclave. In principle, adequate heating is confirmed if the optical density (OD) of the sample is lower than the OD of the thermal evaluation reference standard. In order to get a more detailed impression of the heat treatment, the R-value was calculated by division of the OD of each sample by the OD of the negative control. The decreasing R-values of 6.25, 3.24, 1.59 and 1.2 for the experimentally sterilised pork (the respective beef samples were B-126, B-129, B-132 and B-135) demonstrate the progressive degradation of biological material, i.e. proteins, by increasing process temperatures. For the industrial samples, MBM-1 to MBM-9, R-values of 0.98, 1.08, 0.97, 1.46, 1.45, 1.09, 1.18, 1.28 and 3.49 respectively, were found. By comparison of these results with the R-value of 1.8 for the reference standard, only the experimentally sterilised meat samples B-135 and B-132 and the industrial MBM samples 1–8 can be considered sufficiently sterilised according to the EU legislation ($\geq$133°C, $\geq$3 bar pressure for $\geq$20 min).

Finally all of the autoclaved meat meals and the industrial MBM samples were thoroughly mixed with the plant feed mixture at levels of 50%, 10%, 5% and 1%.

2.2. *Analytical methods*

2.2.1. DNA extraction

From homogenised samples, DNA was isolated using the official German DNA isolation protocol for the detection of genetically modified soya beans (Anonymous 1998) with minor modifications.

A 2 g sample was mixed with 10 ml of cetyltrimethylammoniumbromide (CTAB, Amresco-Biometra, Göttingen, Germany) extraction buffer and incubated at 65°C under agitation for 60 min, followed by the addition of 100 μl of proteinase-K solution (20 mg/ml, Merck Darmstadt, Germany) and then incubated for an additional 2 h at 65°C. In a new micro-centrifuge tube, 1 ml of the aliquot was mixed with 10 μl of newly added proteinase-K solution and finally incubated at 65°C for 60 min; then followed by centrifugation at 6000 *g* for 10 min. About 800 μl of the supernatant were mixed with 600 μl of Ready Red^{TM} (Chloroform–Isoamyl alcohol 24:1, Applichem) solution, then centrifuged at 14000 *g* for 15 min. 600 μl of the supernatant were mixed with 480 μl of isopropanol (Amresco-Biometra, Göttingen, Germany) and incubated at room temperature for at least 30 min, followed by centrifugation at 14000 *g* for 15 min. The supernatant was poured off carefully and the pellets were washed with 500 μl of alcohol (70%, Merck, Darmstadt, Germany), then dried and dissolved in 200 μl of Tris-EDTA-buffer (Biomol, Amresco).

Isolated DNA was finally purified using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

2.2.2. Primers and probes

The first applied PCR assay described by Colgan is specific for bovine mtDNA (Colgan et al. 2001). The forward primer (F) (5′-GCCATATACTCTCCTTGG-3′) and the reverse primer (R) (5′-GTAGGCTTGGGAATAGTA-3′) amplify a fragment of 271 bp, which is detected by the Colgan BOV1 probe: 5′-FAM-CATGACTGACAATGATCTTA-TAMRA-3′. The probes and primers were synthesised by TIB MOLBIOL, Berlin, Germany. The amplification program

includes a primary denaturation for 3 min at 95°C and then 45 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 45 s and extension at 72°C for 30 s.

In the second assay bovine mtDNA was amplified according to Abdel-Fattah (2005) using the primer Bos-109-F (5′-ATCATCGCCTTCTCCACATC-3′) and Bos-109-R (5′-CTTTGAAAAAGGCGTGGG TA-3′) amplifying a fragment of 109 bp.

The amplicon was detected by a TaqMan probe (5′-FAM-ACCAACCTTACC-TAG CTTTCCTCCACA-TAMRA-3′). The design of these oligonucleotides was based on GenBank® accession number V00654. The amplification program was optimised as follows: Primary denaturation for 3 min at 95°C then followed by 45 cycles of denaturation at 95°C for 15 s, primer annealing at 63°C for 30 s and extension at 63°C for 30 s.

The specificity of Bos-109 real-time PCR was previously confirmed by analysis of DNA from different animal (cattle, sheep, goat, pigs, horse, dog, cat, chicken, turkey and fish) and plant species (corn, wheat, barley, oat, soya bean, pea, rice, raps, rye, potato and sunflower seed). The sensitivity was examined by running 10-fold serial dilutions of bovine DNA. The detection limit was found at a dilution of 10^{-7} (0.00001%), i.e. at 116.5 fg. The sensitivity of the assay was further determined by testing a series of DNA isolated from bovine cell cultures containing a microscopically quantified number of cells. The results showed that the PCR assay was able to detect bovine DNA extracted just from a few cells (2.5 cells) or even (but not fully reproducible) a part of a cell (0.25 cell).

Amplification was carried out with the real-time PCR system MX3000P™ (Stratagene) at a final volume of 20 μl for the PCR assay of Colgan, containing 13.35 μl of DNA-free water, 2 μl of PCR-buffer (10x HotMaster Taq buffer with 25 mM Mg^{2+}; Eppendorf, Germany), 0.5 μl of dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP; Roche, Germany), 0.5 μl of probe (4 pmol/μl), 0.5 μl of each primer (10 pmol/μl), 0.15 μl of HotMaster Taq DNA polymerase (5 units/μl; Eppendorf) and 2.5 μl of template DNA. The reaction volume of the Bos-109- PCR assay was composed of 11.8 μl of DNA-free water; 2 μl of PCR-buffer, 0.5 μl dNTP mix, 1.0 μl of probe (4 pmol/μl), 1.0 μl of each primer (10 pmol/μl), 0.2 μl HotMaster Taq DNA polymerase (5 units/μl, Eppendorf) and 2.5 μl template DNA. To prevent carryover contamination, DNA isolation, purification, amplification and post-PCR procedures were carried out in separate rooms. All PCR reactions were carried out in at least triplicate.

3. Results

3.1. Examination of experimentally sterilised beef

The Ct-value of PCR amplification of DNA extracted from autoclaved meat that had undergone heat treatment at 126°C and 2.6 bar pressure (R-value = 6.25) was 18.10 (Table 1). The Ct-value of the subsequent sterilisation at 129°C and 2.8 bar pressure (R-value = 3.24) was 24 indicating a progressive degradation of target DNA. Even in the critical range of the real sterilisation temperature from 132°C (R-value = 1.59) to 135°C (R-value = 1.20), the primers and TaqMan probe of Bos-109 were able to detect the remaining traces of degraded DNA as documented by Ct-values of 24.6 for B-132 and 31.9 for B-135.

In contrast to the Bos-109 real-time PCR, the application of the protocol according to Colgan et al. (2001) only detected bovine meat meals in samples B-129

Table 1. Results of real-time PCR for bovine mitochondrial DNA extracted from feed mixtures containing different levels of experimentally heat-treated beef.

	Heat treatment			Bovine material in the final feed	Method of real-time PCR [Ct-values]	
Sample	Conditions*	R-value[§]	Correctness[#]	mixture [%]	Bos-109	Colgan et al. (2001)
B-135	135°C	1.2	Adequate	100	31.9	no Ct
	3.3 bar			50	no Ct	no Ct
	20 min			10	no Ct	no Ct
				5	no Ct	no Ct
				1	no Ct	no Ct
B-132	132°C	1.59	Adequate	100	24.6	no Ct
	3.1 bar			50	27.3	no Ct
	20 min			10	27.8	no Ct
				5	30.6	no Ct
				1	32.7	no Ct
B-129	129°C	3.24	Inadequate	100	23.0	38.5
	2.8 bar			50	23.7	39.6
	20 min			10	26.5	41.0
				5	27.8	42.5
				1	30.9	no Ct
B-126	126°C	6.25	Inadequate	100	16.5	30.1
	2.6 bar			50	18.2	31.3
	20 min			10	19.6	32.8
				5	20.7	34.0
				1	24.7	37.3

Notes: *Adjustment of the laboratory autoclave used for heat treatment; [§]R-value of the MBM Thermal Evaluation Kit (ELISA-TEK™ PORK M & BM KIT); [#]Correctness of heat treatment according to the results of the MBM Thermal Evaluation Kit.

(sterilised at 129°C and 2.8 bar pressure) and B-126 (sterilised at 126°C and 2.6 bar pressure). The more stringently treated meat samples, B-135 and B-132 treated at 135°C and 132°C respectively, remained negative (Table 1).

In the mixtures of autoclaved meat in plant feedstuffs the results of the PCR showed that the assay Bos-109 was able to detect bovine DNA in all mixtures with the exception of the 135°C-heated beef (Table 1). Comparatively, the real-time PCR assay based on the method of Colgan et al. (2001), only amplified bovine DNA in mixtures of beef sterilised at 126°C and at levels of 50%, 10% and 5% of beef sterilised at 129°C.

3.2. Examination of industrial MBM

The Bos-109 assay was able to detect mixtures of industrial MBM in plant feedstuffs at all concentrations (Table 2), even though MBM-1, MBM-2 and MBM-3 were processed under very stringent conditions. On the other hand, the real-time PCR assay according to Colgan et al. (2001) failed to detect the mixtures of 5% and 1% of MBM-1 and the mixture of 1% of MBM-2. Results for MBM samples 4, 5, 6, 7, 8 and 9 and their respective mixtures in feedstuffs revealed that both real-time PCR assays were able to detect all DNA target sequences (Table 2).

Table 2. Results of real-time PCR for bovine mitochondrial DNA extracted from feed mixtures containing different levels of industrial meat and bone meal (MBM) samples.

Sample	Heat treatment			Industrial MBM in the final feed mixture [%]	Method of real-time PCR [Ct-value]	
	Conditions*	R-value[§]	Correctness[†]		Bos-109	Colgan et al. (2001)
MBM-1	137°C 3.4 bar 24 min	0.98	Adequate	100	29.6	41.3
				50	30.3	43.1
				10	34.1	42.0
				5	35.1	no Ct
				1	36.3	no Ct
MBM-2	137°C 3.4 bar 24 min	1.08	Adequate	100	30.1	38.8
				50	30.8	41.4
				10	34.6	42.1
				5	34.9	44.1
				1	35.6	no Ct
MBM-3	137°C 3.4 bar 24 min	0.97	Adequate	100	26.5	34.9
				50	28.8	38.6
				10	31.7	38.9
				5	33.5	39.0
				1	36.6	41.4
MBM-4	Records not available[#]	1.46	Adequate	100	29.3	32.8
				50	27.3	39.2
				10	30.6	36.0
				5	32.0	36.8
				1	33.9	39.0
MBM-5	Records not available[#]	1.45	Adequate	100	28.0	33.0
				50	30.2	39.3
				10	32.0	36.4
				5	32.4	37.4
				1	35.2	37.0
MBM-6	Records not available[#]	1.09	Adequate	100	28.0	34.5
				50	28.8	38.0
				10	30.7	35.3
				5	31.8	37.5
				1	32.2	37.0
MBM-7	Records not available[#]	1.18	Adequate	100	27.3	34.9
				50	28.6	38.8
				10	29.9	36.3
				5	31.8	39.0
				1	34.4	38.2
MBM-8	Records not available[#]	1.28	Adequate	100	25.8	30.3
				50	26.7	34.4
				10	29.1	34.3
				5	31.7	36.7
				1	33.2	37.7
MBM-9	Records not available[#]	3.49	Inadequate	100	25.3	29.4
				50	26.6	34.2
				10	27.8	38.7
				5	30.1	35.6
				1	31.1	40.0

Notes: *Parameter of heat treatment according to technical process records; [§]R-value of the MBM Thermal Evaluation Kit (ELISA-TEK™ PORK M & BM KIT); [†]Correctness of heat treatment according to the results of the MBM Thermal Evaluation Kit; [#]Processed according to the EU legislation 99/534/EC due to technical process records but detailed data of temperature and pressure were not available.

In principle, Ct-values for all samples of experimentally sterilised beef and industrial MBM samples were considerably lower for the Bos-109 assay than for the assay of Colgan et al. (2001).

4. Discussion

For the control of BSE transmission through animal feedstuffs contaminated with its causative agent, the scrapie-form of the prion protein (Prp^{Sc}), highly sensitive and reliable methods for the detection of bovine MBM are essential. In the present study, we compared the ability of two real-time PCR-based methods (Abdel-Fattah 2005 and Colgan et al. 2001) to detect bovine mtDNA in experimentally sterilised meat, in samples of MBM industrially produced in Germany and finally in mixtures of these materials with plant feedstuffs.

A precondition for sensitive PCR assays detecting heat fragmented DNA is the use of target sequences which occur in high copy numbers per cell. In general, single copy targets deliver insufficient results compared to multi-copy targets as shown by Abdel-Fattah (2005). Therefore, bovine mtDNA was chosen as target sequence because of the occurrence in muscles of more than 1000 copies per cell, and the possibility for the design of a PCR-assay characterised by an appropriate analytical specificity.

It is strongly assumed that Prp^{Sc} is inactivated when the animal material is treated for 20 min at 133°C and 3 bar pressure under wet sterilisation conditions (von Holst et al. 2000). For this reason, the beef samples in this study were sterilised in the laboratory at temperatures above and below this limit to detect the effect of temperature on DNA fragmentation and its influence on PCR amplification. Generally, DNA is a suitable target for species identification because of its relative heat stability and the higher degree of specificity compared to protein structures (Colgan et al. 2001; Mendoza-Romero et al. 2004).

The efficiency and correctness of heat treatment according to European legislation can be examined by the ELISA-based MBM Thermal Evaluation Kit (von Holst et al. 2000). In the present study, the examination of experimentally sterilised meat revealed R-values of 1.2 and 1.59 for samples treated at autoclave settings of 135°C and 132°C respectively. With respect to the conclusions of Unglaub (1997) and von Holst et al. (2001), R-values of lower than 2.0 indicate compliance with the European minimum sterilisation conditions. This means that both experimentally autoclaved meat samples B-135 and B-132 were efficiently sterilised according to the European legislations.

Results of the Bos-109 assay (amplifying a 109 bp DNA segment) revealed a higher sensitivity in the detection of bovine mtDNA in all sterilised meat samples (Table 1). The Bos-109 PCR was positive even after heat treatment at 135°C and a pressure of 3.3 bar. The mixtures of experimentally autoclaved meat with plant feed were also detectable, with the exception of those of the 135°C level (Table 1). The Bos-109 assay also showed a high sensitivity in the examination of mixtures with the commercial MBM, including the highly processed MBM-1 and MBM-2. Compared with the experimentally sterilised beef undergoing an analogous heat treatment according to autoclave settings, the higher sensitivity for mixtures of industrial MBM and results of the MBM Thermal Evaluation test indicate the significant differences in the biological action of high temperatures in dependence of the reaction environment.

In comparison, the PCR assay of Colgan et al. (2001) that amplified a 271 bp DNA segment was less sensitive in detection of heat-treated meat (132°C and 135°C) and its mixtures with plant feed. Even the 1% mixture of beef treated at 129°C with plant feed was undetectable. The Ct values of all the samples of both the experimentally sterilised beef and industrial MBM were at least 10 Ct lower in the Bos-109 assay than for the PCR according to Colgan et al. (2001), approving the considerably higher sensitivity of the Bos-109 assay especially for tissues processed under high temperature and pressure. Furthermore, the observed Ct values of the PCR according to Colgan et al. (2001), were often higher than 40 and therefore close to the detection threshold. In practical application, low reproducibility and reliability would have to be expected for that assay.

The main cause of higher sensitivity is the short amplicon of 109 bp in the Bos-109 assay. Under high temperature treatment, DNA is degraded into shorter fragments (Momcilovic and Rasooly 2000). An increase in the treatment temperature is correlated with a significant reduction in the quantity and size of the extracted DNA (von Holst et al. 2004; Abdel-Fattah 2005). For this reason, amplification of target sequences shorter than 150 bp was found to be essential for the successful amplification in samples with highly degraded DNA (Martín et al. 2007). In a previous study by Frezza et al. (2003), only primers amplifying short fragments of mtDNA were able to detect MBM that was treated under high temperature and pressure. Although Frezza et al. (2003) examined only crude MBM, they concluded that a PCR-based method designed to amplify relatively short sequences could possibly be used for the successful analysis of MBM and feeds.

The results of the present study supports the previous conclusions (Momcilovic and Rasooly 2000; Frezza et al. 2003; Abdel-Fattah 2005; Chiappini et al. 2005; Martín et al. 2007; Frezza et al. 2008) that the amplification of short DNA fragments ensures a more sensitive detection of MBM in feedstuffs, especially when these MBM had been treated under stringent processing conditions. Similar results were also reported by Kingombe et al. (2001).

The general requirement of short amplicons is additionally emphasised by the results of Matsunaga et al. (1999) and Wolf et al. (1999). Although these authors had analysed MBM treated under mild conditions (<128°C and <2.6 bar pressure, R value $= 2.1$), they found negative results for the amplification of relatively long DNA segments (274 bp and 464 bp respectively).

According to the aims of the present study, we examined the addition of MBM at a minimum of 1%. The reported Ct values for both pure and diluted industrial MBM demonstrate that the detection level of the Bos-109 real-time PCR is obviously below 1% and can possibly fulfil the requirement for a detection limit of 0.1%. Taking into consideration that the reproducibility of TaqMan real-time PCR decreases from Ct values higher than about 40, the precondition for a detection limit of 0.1% is a Ct value of below 30 for pure MBM. Chiappini et al. (2005) found Ct values for pure MBM in the same range compared with the results of this study. When they raised the treatment temperature from 133 to 137°C, they observed an increase in Ct values from 25.63 to 30.96. This indicates a significant decrease in the amplifiable target DNA in correlation with treatment temperature. With respect to the reported Ct value of 30.96 for pure MBM sterilised at 137°C, the reliable detection of concentrations as low as 0.1% of such highly processed MBM remains a challenge as already stated by von Holst et al. (2004). With respect to the unknown conditioning of MBM possibly mixed to composed feed, the temperature-dependent

degradation of target-DNA also counteracts attempts for further quantitative examinations.

In contrast with these studies, other reported PCR assays (Tartaglia et al. 1998 and Wang et al. 2000; Krčmář and Renčová 2001; Myers et al. 2001) were able to detect lower levels of MBM (up to 0.125%) in feedstuffs or in mixtures of other animal species. These authors neither examined the processing method of the MBM samples nor determined the biological effectiveness of heat treatment, for instance by the determination of the R value using the MBM Thermal Evaluation test. Consequently, these reports lack an impression of the extent of bovine DNA degradation due to heat treatment. In recent reports, detection limits of 0.1 and 0.05% bovine MBM were found (Prado et al. 2007, 2009). MBM was produced with steam pressure sterilisation at 133°C, 3 bar for 20 min (Prado et al. 2007) and 134°C for 20 min (Prado et al. 2009). In spite of the well documented process conditions, the estimation of the alteration of the raw material is missing in these studies too.

Besides the possibly limiting effect of the sterilisation temperature, the complex conditions of the whole rendering process have to be considered. A process with a sterilisation temperature of 133°C may result in greater damaging effect on DNA than another one performing sterilisation at an even higher temperature (von Holst et al. 2004). Owing to the possibly different effectiveness of sterilisation in rendering plants and the correlated biological effects (Gaede 1998; Weber et al. 1998), the validation of each PCR assay must include the analysis of the material treated under defined and increased variety of processing conditions. Without this precondition, the reliability of PCR assays for MBM detection is doubtful.

Von Holst et al. (2004) suggested that for ensuring a detection level of $<0.5\%$ MBM the temperature threshold range of 136–138°C should not be exceeded. With respect to the fact that the European legislation only defines the minimum requirements for MBM treatment, more intensively sterilised MBM has to be considered. Especially for amplicons longer than 150 bp, the present results indicate that sufficient reliability of real-time PCR cannot be assured if MBM was treated at much more stringent conditions than the legal procedure of at least 133°C for $\geq$20 min and a pressure of $\geq$3 bar. Taking into consideration the unknown treatment, and therefore the unpredictable alteration of supposed industrial MBM, the protocols for examination of composed bovine feed have to use amplicons that are as short as possible.

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