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A PCR-based test for species-specific determination of heat treatment conditions of animal meals as an effective prophylactic method for bovine spongiform encephalopathy

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Abstract

Efficiency of animal waste sterilization prescribed by the European Union and Switzerland was verified using a pork-based ELISA and two PCR assays ($tRNA^{Glu}$/cytochrome b specific for vertebrates; bovine species-specific cytochrome b mitochondrial genome). A total of 204 samples of feedingstuffs were analysed including reference materials subjected to known heat treatments. Both ELISA and PCR assays were able to detect poorly heat-treated feedingstuffs if there was enough pork-based material present. The proposed species-specific PCR test, however, showed a higher sensitivity and specificity as it specifically detected bovine material. Nevertheless, the PCR assay will not detect bovine material in properly heat-treated feeds as the DNA is too fragmented. It is, however, very useful as a rapid, sensitive, and specific method for the routine screening of animal meals with regard to prophylaxis of BSE.

Keywords: BSE; ELISA; PCR; Animal meals

1. Introduction

To ensure the exclusion of the bovine spongiform encephalopathy (BSE) agent and other pathogens from animal feeds the European Union (EU) specifies specific treatment conditions in (Decision 96/449 EC; Anon., 1996). In order to guarantee the denaturation of hazardous agents in animal waste products and be considered free of BSE in particular the meat and bone meal (MBM) manufacturers must heat these products at 133°C for 20 min at a pressure of 3 bar (Anon., 1993, 1997).

To meet these criteria, imported animal feedstuffs have to pass an ELISA based test (Anon., 1994, Hofmann, 1997). This test involves the detection of non denatured pork proteins in meat meal and MBM and has been evaluated in a collaborative study involving 21 laboratories from 12 European countries (Unglaub, Müller, Jemmi & Stuker, 1999).

The ultimate aim of the heat treatment is the elimination of possible BSE agents from animal waste materials. It is possible that an animal feedstuff made exclusively using non-pork animal waste materials, and which has not been treated according to the prescribed conditions could pass the ELISA pork-based antibody test. Also, using the ELISA test will not guarantee the non-adulteration and safety of fish-based feedstuffs by animal waste ingredients.

Several techniques including DNA hybridization (Baur, Tafel-Greiding & Leibhardt, 1987, Chikuni, Ozutsumi, Koishikawa & Kato, 1990; Ebbehoj & Thomsen, 1991), polymerase chain reaction (PCR) (Chikuni, Tabata, Kosugiyama, Momma & Saito, 1994; Fei, Okayama, Yamanoue, Nishikawa, Mannen & Tsuji, 1996; Wolf, Rentsch & Hübner, 1999), DNA sequencing (Chikuni, Tabata, Saito & Monma, 1994) or immunological techniques (e.g. immunodiffusion, countercurrent immunoelectrophoresis), as well as isoelectric focusing (Wintero, Thomsen & Davies, 1990) have been used for the identification of the species in meat and meat products. Recently a rapid and simple polymerase chain reaction (PCR) method for species specific

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identification of meat species from cooked meat and meat products has been described (Matsunaga et al., 1999). By means of a simple or multiplex PCR, this method is able to detect an individual species in a mixture of cooked animal meat materials.

All these species-specific test methods were focused on meat and meat products for commercial, economic, ethnic or religious reasons and not because of prophylactic concerns.

In the present work, the pork antibody ELISA-based test is compared to two PCR methods (Matsunaga et al., 1999; Wolf et al., 1999) to determine their usefulness to ensure adequate heat treatment of both meat and MBM meals.

2. Materials and methods

2.1. Samples

A total of 204 samples was used in this study including eight animal meat meal (AM) samples from the European Commission, Joint Research Centre, Environment Institute, Food & Drug Analysis, Consumer Protection Unit, Ispra (VA), Italy, which were used as reference materials in a previous validation study of the ELISA test (Unglaub et al., 1999); 15 imported fish meals obtained from the Swiss Border Veterinary Services; 166 meat and MBM meals, compound feedingstuffs obtained from three main Swiss manufacturers (manufacturer A: 77, manufacturer B: 69, and manufacturer C: 20 samples) and 15 compound feedingstuffs from the Swiss Federal Research Station for Animal Production, Feed Inspection Unit, Posieux, Switzerland.

2.2. ELISA test

The EU recommended ELISA method (Cortecs Diagnostics Limited, Deeside, UK) using the pork antibody-based test was performed as described in the manufacturer's protocol.

2.3. DNA extraction

The High Pure PCR Template Preparation kit (Roche Diagnostics, Cat. No. 1 796 828) was used for the extraction of DNA from 50 mg of meat-and-bone meal samples according to the manufacturer's instructions for the extraction of DNA from tissue. Five μl of the eluted DNA were used as a template for DNA amplification.

2.4. Polymerase chain reaction amplifications

Two PCR amplification methods were used in this work. The first method (Wolf et al., 1999) amplifies a specific fragment of 464 bp which is part of the mitochondrial genome ($tRNA^{Glu}$/cytochrome b) using L14735 (5′-AAA AAC CAC CGT TGT TAT TCA ACT A- 3′) as forward oligo primer and H15149 (5′-GCC CCT CAG AAT GAT ATT TGT CCT CA- 3′) as reverse oligo primer. The second method (Matsunaga et al., 1999) detects a bovine species-specific amplicon of 274 bp from the bovine mitochondrial genome using SIM (5′-GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA- 3′) as forward oligo primer and SIM/B for bovine species (5′-CTA GAA AAG TGT AAG ACC CGT AAT ATA AG-3′) as reverse primer.

The PCR amplifications were carried out in 50 μl of PCR mixture containing 5 μl of template DNA, 8 μl of 0.2 mM of each deoxynucleoside triphosphate mixture (Roche Diagnostics AG, Rotkreuz, Switzerland), 5 μl of 10×PCR buffer II (QIAGEN AG, Basel, Switzerland), 5 μl of 25 mM of $MgCl_2$ (QIAGEN AG, Basel, Switzerland), 0.25 μl of 200 μM solution of each forward and reverse primers (1 μM final concentration) (MWG-Biotech GmbH, Ebersberg, Germany), 0.10 μl of Tween 20 (2% final concentration) (Sigma Switzerland, Buchs, Switzerland), 0.25 μl of 5 U/μl (1.25 unit final concentration) of HotStartTAq™ (Qiagen AG, Basel, Switzerland), and 26.15 μl of sterile double distilled water. The master mix for the bovine species-specific mitochondrial genome (Matsunaga et al., 1999) was prepared as described for the mitochondrial genome ($tRNA^{Glu}$/cytochrome b) but using its indicated primers. The PCR master mixes were prepared in a sterile laminar air flow to produce approx. 100 tubes of 45 μl each, containing all the ingredients except the DNA template, the tubes were at −20°C until needed.

The original amplification programs were optimized and converted for the GeneAmp PCR system machine model 9600 (Perkin-Elmer, Rotkreuz, Switzerland). The amplification program for the mitochondrial genome ($tRNA^{Glu}$/cytochrome b) was optimized as follows: A cycle of denaturation for 15 minutes at 96°C, 30 cycles of melting at 96°C for 20 s, annealing at 55°C for 40 s, extension at 72°C for 40 s, and a final elongation step at 72°C for 10 min with a total running time of 2.4 h. The PCR program for the bovine species-specific amplicon was optimized as follows: A cycle of denaturation for 15 min at 95°C, 30 cycles of melting at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, and a final elongation step at 72°C for 10 min with a total running time of 2.2 h.

The PCR amplicons were separated electrophoretically by loading a total volume of 20 μl of amplicon including 6 μl of stop solution buffer onto a 2.5% agarose gel (pulsed field certified agarose, Bio-Rad, Glattbrugg, Switzerland). The electrophoresis was performed in 0.5 TBE (Tris–borate-EDTA buffer/double distilled water) for 1 h at 100 volts. Amplicons were

Table 1
ELISA and PCR results obtained with samples subjected to known heat treatment conditions and of known composition (for setting up the PCR method)

Sample	Origin of sample	Heat treatment conditons	Composition[a]	ELISA[b] (*R*-value)	PCR[c] (464 bp)	PCR[d] (274 bp)	ASA[e] (%)
AM[f] I	EU[g] ELISA validation study	20 min, 133–135°C, 3.0–3.2 bar[h]	Meat meal	1.10	–	–	N. D[i]
AM I	EU ELISA validation study	20 min, 133–135°C, 3.0-3.2 bar	Meat meal	1.10	–	–	N.D.
AM II	EU ELISA validation study	20 min. 128–130°C, 0.6–2.8 bar	Meat meal	1.70	–	–	N.D.
AM II	EU ELISA validation study	20 min, 128–130°C, 2.6–2.8 bar	Meat meal	1.70	–	–	N.D.
AM III	EU ELISA validation study	20 min, $T<128$°C, $P<2.6$ bar	Meat meal	2.10	–	–	N.D.
AM III	EU ELISA validation study	20 min, $T<128$°C, $P<2.6$ bar	Meat meal	2.10	–	–	N.D.
AM IV	EU ELISA validation study	20 min, $T>133$°C, no overpressure [j]	Meat meal	13.00	+	+	100
AM IV	EU ELISA validation study	20 min, $T>133$°C, no overpressure	Meat meal	13.00	+	+	99
FM[k] I	Denmark	N.G.[l]	Fish meal	0.78	+	–	N.D.
FM II	Denmark	N.G.	Fish meal	0.73	+	–	N.D.
FM III	Denmark	N.G.	Fish meal	0.81	+	–	N.D.
FM IV	Denmark	N.G.	Fish meal	1.10	+	–	N.D.
FM V	Germany	N.G.	Fish meal	0.80	+	–	N.D.
FM VI	Germany	N.G.	Fish meal	0.76	+	–	N.D.
FM VII	Germany	N.G.	Fish meal	1.20	+	–	N.D.
FM VIII	Norway	N.G.	Fish meal	1.10	+	–	N.D.
FM IX	Norway	N.G.	Fish meal	1.10	+	–	N.D.
FM X	Norway	N.G.	Fish meal	1.10	+	–	N.D.
FM XI	Norway	N.G.	Fish meal	1.10	+	–	N.D.
FM XII	Norway	N.G.	Fish meal	1.10	+	–	N.D.
FM XIII	Norway	N.G.	Fish meal	1.10	+	–	N.D.
FM XIV	Norway	N.G.	Fish meal	1.10	+	–	N.D.
FM XV	Norway	N.G.	Fish meal	1.20	+	–	N.D.
CF[m] I	Switzerland	N.G.	V. F.[n]	6.1	–	–	N.D.
CF II	Switzerland	N.G.	V. F.	7.7	N.S.A.[o]	N.S.A.	N.D.
CF III	Switzerland	N.G.	V. F. & A. M. F.[p] traces	5.2	–	N.D.	N.D.
CF IV	Switzerland	N.G.	V. F. & A. M. F. traces	3.5	–	N.D.	N.D.
CF V	Switzerland	N.G.	V. F. & A. B. F.[q] (0.2%)	7.5	–	N.D.	N.D.
CF VI	Switzerland	N.G.	V. F. & A. B. F. traces	4.4	N.S.A.	N.S.A.	N.D.
CF VII	Switzerland	N.G.	V. F. & A. M. F & A. B. F. (0.09%)	11.8	N.S.A.	–	N.D.
CF VIII	Switzerland	N.G.	V. F. & A. M. F & A. B. F. (0.026%)	7.7	–	N.D.	N.D.
CF IX	Switzerland	N.G.	V. F. & A. B. P & F. M. F.[r] traces	3.9	–	N.D.	N.D.
CF X	Switzerland	N.G.	V. F. & A. B. P & F. M. F. (0.14%)	4.3	+	–	N.D.
CF XI	Switzerland	N.G.	V. F. & A. B. P & F. M. F. (0.61%)	8.1	N.S.A.	N.D.	
CF XII	Switzerland	N.G.	A. M. F. (0.20%)	6.8	–	N.D.	N.D.
CF XIII	Switzerland	N.G.	A. M. F. (0.26%)	3.4	+	–	N.D.
CF XIV	Switzerland	N.G.	V. F. & A. M. F & A. B. F. (0.80%)	9.6	+	+	N.D.
CF XV	Switzerland	N.G.	V. F. & A. M. F & A. B. F. (0.70%)	5.7	+	+	N.D.

[a] For CF samples, the animal or fish muscle and bone % were calculated according to the microscopy analysis method at the Swiss Federal Research Station for Animal Production, Feed Inspection Unit, Posieux.
[b] Enzyme linked immunosorbant assay *R*-value; according to the ELISA kit manufacturer, a *R*-value greater than 3.1 means the heat treatment conditions were not respected. *R*-value is the optical absorbance of the sample/optical absorbance of negative control.
[c] Polymerase chain reaction for the detection of mitochondrial DNA according to Wolf et al. (1999) giving an amplicon of 464 base pairs.
[d] Polymerase chain reaction for the species-specific identification of cattle DNA sequence according to Matsunaga et al. (1999) giving an amplicon of 274 base pairs.
[e] Amplicon sequence analysis (ASA); amplicon sequence alignment identity to GenBank *Bos taurus* complete mitochondrial genome sequence, accession number emb V00654.
[f] Animal meal, samples provided by the Joint Research Centre Laboratory, Environment Institute, Food & Drug Analysis, Consumer Protection Unit, Ispra (VA), Italy.
[g] European Union.
[h] Wet sterilization.
[i] Not done.
[j] Dry sterilization.
[k] Fish meal, imported from respective country.
[l] Not given.
[m] Compound feedingstuffs.
[n] Vegetal feedingstuffs.
[o] Non specific amplicon.
[p] Animal muscle fiber.
[q] Animal bone fragments.
[r] Fish muscle fiber.

visualized with ultraviolet light after soaking the agarose gel for 15 min in a 0.5 g/ml ethidium bromide solution (Bio-Rad, Glattbrugg, Switzerland).

2.5. Sensitivity of the PCR system

The screening of the sensitivity of the PCR method was carried out by running PCR of a serial lots of negative fish (FM II with a *R*-value of 0.73) and meat (AM I with a *R*-value of 1.1) samples contaminated respectively with 0.1, 0.5, 1.0, 5.0, 10.0, and 50.0% of the positive sample AM IV with a *R*-value of 13.

2.6. Amplicon sequence determination

The bovine specific 274 bp PCR products were purified and concentrated by using the Microcon YM-50 filter (Cat. No. 42416, Millipore AG, Volketswil, Switzerland) according to the manufacturer's instructions with a minor modification. Briefly, 30 μl of amplicon were loaded in the YM-50 filter column and washed twice with 500 μl of sterile double distilled water and spinning at $14{,}000\times g$ for 24 min at 4°C using a Eppendorf centrifuge model 5417R (Eppendorf AG, Germany), the purified DNA was collected using 50 μl of sterile double distilled water by spinning at $4000\times g$ for 6 min.

For the characterization of the 274 bp PCR amplicon, the cycle sequencing reaction mixture was prepared using the Big Dye Terminator Cycle Sequencing ready reaction kit (Cat. No. 4303149, PE Applied Biosystems, Perkin-Elmer, Rotkreuz, Switzerland). Excess terminators were removed using DyeEx Spin column (Cat. No. 63106, Qiagen AG, Basel, Switzerland) before preparing and loading the samples onto the ABI Prism 310 Genetic Analyser system (Applied Biosystems, Rotkreuz, Switzerland) as specified in the manufacturer's protocol. The obtained sequences were aligned to GenBank sequences.

3. Results

3.1. ELISA test

The *R*-values of the six reference samples from the EU study which satisfied the requirements of heat treatment (wet sterilization at 133°C for 20 min at 3 bar) (AM I–III) were all below the threshold value of 3.0, ranging from 1.1 to 2.1 (Table 1). The two remaining samples (AM IV) which did not fulfill the heat treatment conditions showed a high *R*-value of 13 (Table 1). The *R*-values of imported fish meals were low ranging between 0.73 and 1.20 (Table 1). The feedingstuffs obtained from Swiss Federal Research Station for Animal Production, Feed inspection unit, produced high *R*-values between 3.4 and 11.8 (Table 1). Among the samples obtained from the Swiss feedingstuffs companies 17 (10.2%) of 166 presented high *R*-values ranging between 3.0 and 12.3 (Table 2). The remaining

Table 2
Results of ELISA and PCR tests on feedingstuffs collected from the main rendering companies in Switzerland

Origin	Composition	*R*-value range[a]	No. of sample	+464 bp[b]	+247 bp[c]	No. and identity of ASA (%)[d]
Company A	Meat meal	0.8–2.9	53	1	1	N.D.[e]
Company A	Meat meal	3.0–4.0	4	2	2	N.D.
Company A	MBM[f]	0.7–1.1	8	8	8	3 (96–99%)
Company A	MBM	12.1–12.3	2	2	2	2 (95–99%)
Company A	CF[g]	0.9–2.2	9	2	2	N.D.
Company A	CF	3.1	1	0	0	N.D.
Total			77	15	15 (19.5%)	
Company B	Meat meal	1.0–2.9	59	16	16	4 (94–97%)
Company B	Meat meal	3.0–6.2	10	10	10	9 (96–100%)
Total			69	26	26 (37.7%)	
Company C	Meat meal	1.1–1.7	15	0	0	N.D.
Company C	MBM	1.1–1.2	5	0	0	N.D.
Total			20	0	0 (0%)	
Gross total			166	41	41 (25%)	18 (94–100%)

[a] Enzyme linked immunosorbant assay *R*-value; according to the ELISA kit manufacturer, a *R*-value greater than 3.0 means the heat treatment conditions were not respected. *R*-value is the optical absorbance of the sample/optical absorbance of negative control (Hofmann, 1997).

[b] Polymerase chain reaction for the detection of mitochondrial DNA according to Wolf et al. (1999) giving an amplicon of 464 base pairs.

[c] Polymerase chain reaction for the species-specific identification of cattle DNA sequence according to Matsunaga et al. (1999) giving an amplicon of 274 base pairs.

[d] Number of samples used and amplicon sequence alignment (ASA) identity (%) to GenBank *Bos taurus* complete mitochondrial genome sequence, accession number EMBL V00654.

[e] Not done.

[f] Meat and bone meal.

[g] Compound feedingstuffs.

samples 149 (89.8%) presented *R*-values ranging between 0.70 and 2.9 (Table 2).

3.2. Polymerase chain reaction tests

3.2.1. Mitochondrial genome (464 bp amplicon) PCR test

Of the EU reference samples, only two (AM IV) were positive, the other samples (AM I–III) were all negative for this test (Table 1). All the 15 fish meal samples (FM) were positive for this DNA fragment (Table 1). Among the samples from the Swiss feedingstuffs industries (companies A–C), 41 (25%) of 166 were positive for this test, 15 (19.5%) of 77 from company A, and 26 (37.7%) of 69 from company B (Table 2). None of the 20 samples from company C was positive for this fragment (Table 2).

3.2.2. Bovine species-specific mitochondrial cytochrome b gene (274 bp) PCR test

The PCR amplification for the bovine species-specific mitochondrial cytochrome b gene of the AM I–III reference samples which were heat treated according to Swiss and EU prescriptions were all negative (Table 1), but samples AM IV which did not satisfy the requirements were strongly positive (Table 1). The fish meal samples were all negative for the bovine species-specific DNA (Table 1). Of the 166 samples from the Swiss feedingstuffs industries, 41 (25%) were positive (Table 2). All positive samples for the 464 bp amplicon were also positive for this test except two compound feedingstuffs samples from the Swiss Feed Inspection Unit Laboratory which were found to be negative (Table 1).

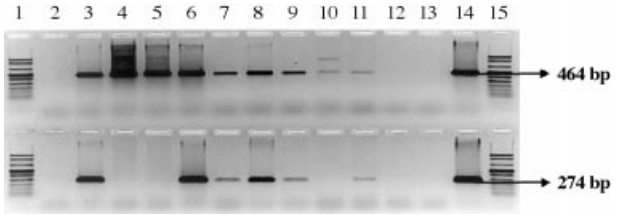

Fig. 1. Typical PCR results of reference, fish, MBM, meat meal, and compound feedingstuffs samples. PCR amplicons of mitochondrial cytochrome b (464 bp) and bovine species-specific mitochondrial cytochrome b gene PCR amplicon (274 bp) on 2.5% (pulsed field certified agarose, Bio-Rad, Glattbrugg, Switzerland). Upper lanes, 464 bp amplicon of mitochondrial cytochrome b gene: Lanes 1 and 15, DNA size marker VIII (Roche Diagnostics, Cat. No: 1336 045); lane 2, MBM meal, a reference sample from EU ELISA test kit evaluation study (20 min, $T > 133$°C, 3 bar) with *R*-value of 1.1; lane 3, MBM meal, a reference sample from EU ELISA test kit evaluation study (20 min, $T < 133$°C, no overpressure) with *R*-value of 13; lane 4, fish meal imported from Denmark with *R*-value of 0.81; lane 5, fish meal imported from Germany with *R*-value of 2.9; lane 6, MBM from company A, Switzerland, with a *R*-value of 12.3; lane 7, meat meal from company A, Switzerland, with a *R*-value of 1.7; lane 8, meat meal from company B, Switzerland, with a *R*-value of 6.2; lane 9, meat meal from company B, Switzerland, with a *R*-value of 2.8; lane 10, compound feedingstuffs for farm animal, Switzerland, with a *R*-value of 8.1 presenting non specific bands for the 464 bp amplicon due probably to vegetal DNA but negative for the 274 bp amplicon; lane 11, compound feedingstuffs for farm animal, Switzerland, with a *R*-value of 5.7; lane 12, negative control (lysis buffer from the DNA extraction kit); lane 13, PCR master mixture without any DNA sample; lane 14, positive control, DNA extracted from beef tissue. Bottom lanes, same samples but amplifying the bovine species-specific 274 bp amplicon from cytochrome b gene.

3.2.3. Sensitivity of the PCR system

The results of the sensitivity test showed that a PCR positive signal could be detected from both negative fish or meat meal samples containing as low as least 0.5% of positive AM IV sample (Fig. 2).

3.2.4. Amplicon sequence determination

The alignment to the GenBank database of the 20 amplicon positive for the bovine species-specific 274 bp amplicon and subjected to ASA including two AM IV samples (Table 1) and 18 samples from the Swiss rendering companies (Table 2) gave agreements, ranging from 94 to 100%, with the *Bos taurus* complete mitochondrial genome sequence accession number EMBL V00654.

Typical results of some reference samples, fish meal, meat meals, MBM meals as well as compound feedingstuffs and the sensitivity tests are illustrated in Figs. 1 and 2.

4. Discussion

Since the emergence of BSE in Europe, the use of non heat-treated bovine based materials in animal feeding-

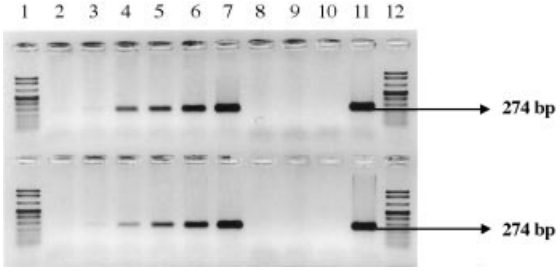

Fig. 2. Sensitivity screening of PCR method for adulteration of animal meals with non correctly heat treated animal meal. Bovine species-specific mitochondrial cytochrome b gene amplicon (274 bp) amplified from contaminated contaminated AM I and FM II negative samples with positive reference sample AM IV on 2.5% agarose gel (pulsed field certified agarose, Bio-Rad, Glattbrugg, Switzerland). Upper lanes: results of contamination of negative animal meal sample (AM I: ELISA *R*-value 1.1) with positive animal meal sample (AM IV: ELISA *R*-value 13.0); lanes 1 and 12 size marker VIII (Roche Diagnostics, Cat. No. 1336 045); lane 2, contamination with 0.1%; lane 3, contamination with 0.5%; lane 4, contamination with 1%; lane 5, contamination with 5%; lane 6, contamination with 10%; lane 7, contamination with 50%. Bottom lanes; results of contamination of negative fish meal sample (FM II: ELISA *R*-value 0.73) with positive animal meal sample (AM IV: ELISA *R*-value 13.0). Contamination procedure as in upper lanes.

stuffs has been suggested as the cause of the spread of the infectious agents. The introduction and regulation of heat treatment conditions for animal wastes was set up to reduce the risk of transmission of the BSE agents by animal meals of bovine origin.

The results obtained from this investigation using the reference samples from the EU joint evaluation study of the ELISA kit (von Holst, Anklam & Vnglaub, 1999) demonstrates the usefulness of using bovine species-specific identification to determine of the heat treatment conditions for bone and meat meals, fish meals, meat meals, and compound feedingstuffs for farm animals (see Table 1). The reference samples which had been adequately heat treated, as shown by their low *R*-values (less than 3), were also negative for both PCR (274 and 464 bp amplicons) tests. However, for the reference samples for which the conditions were not adequate (AM IV) and had a high *R*-value of 13, which were also strongly positive for the presence of the two DNA fragments (Table 1 and Fig. 1). The alignment of the obtained sequences from the AM IV samples confirmed their bovine origin. This means that when heat treatment is adequate, proteins and DNA materials in the samples are destroyed and both ELISA and PCR tests are negative. This may be the case for company C, since none of the bone and meat or meat meals from this company was positive.

The fish meals which are treated differently to meat waste materials gave low *R*-values of 0.73–1.20 demonstrating the absence of pork antigens in these samples. Nevertheless, the PCR test for the animal mitochondrial cytochrome b gene (464 bp amplicon) was strongly positive for these samples and negative for the bovine species-specific PCR (274 bp amplicon). This confirms the specificity of both the ELISA and PCR tests. In this context, one can assume that adulteration of fish meal with poorly heat treated animal meal, especially beef or pork, will increase the *R*-value and so can be detected by the PCR tests. When the adulteration of fish meal is made with properly heat-treated animal ingredients both methods will fail because animal proteins and DNA are highly denatured and fragmented respectively. Nevertheless, as these products have been adequately heat treated, they can be considered safe.

The application of the ELISA test to the different samples collected from feedingstuffs industries indicated that all samples with *R*-values higher than 3.0; e.g. 17 (10.2%) of 166 samples were also positive for the PCR tests except for two samples with *R*-values of 3.2 and 4.0 (Table 2: company A). It is known that primers used to amplify the 464 bp amplicon do not detect horse or poultry mitochondrial cytochrome b gene (Ch. Wolf, pers. comm.) and thus this suggests that the two particular samples were made exclusively with poultry or horse waste materials. It is interesting to note that out of the 166 industrial samples tested with the ELISA kit 27 (16.3%) gave *R*-values below the threshold value of 3.0 ranging between 0.7 and 2.9 but were still positive with the PCR tests. This demonstrates the inability of the ELISA test to detect non pork-based material or animal meals containing less than 10% of pork material (von Holst et al., 1999). It may be also true for poorly heat-treated animal waste because the bovine species-specific DNA amplicon sequences from some of these samples all aligned, with identities ranging from 94 to 100%, with the bovine cytochrome b mitochondrial gene of the GenBank database. With such low *R*-values, these samples could pass as being adequately heat treated. This suggests that the pork-based antibody ELISA kit only defects samples containing significant amounts of pork material. Samples which contain only beef, fish or species other than pork will react weakly and show low *R*-values to give false negative results.

The results obtained with all 15 compound feedingstuffs (CF) showed high *R*-values ranging from 3.4 to 11.8 (Table 1), but only four were found to be positive for the 464 bp amplicon and two were positive for the 274 bp amplicon. According to the Feed Inspection Unit Laboratory, microscopic analysis of these four samples revealed that the two samples which were positive for the 274 bp amplicon contained 0.70 and 0.80% of bone and muscle fiber fragments, the two other positive samples for the 464 bp amplicon contained 0.14 and 0.26% of fish bone and muscle fibers. These results indicate the high specificity and sensitivity of the PCR systems. The high *R*-values of these feedingstuffs show the presence of an antigen cross reacting with the pork-based antibodies from the ELISA test, indicating the inappropriate use of the ELISA kit for these kinds of sample.

In a recent study, Detsch, Homikel and Schwägele (1999) found that the pork antibody-based ELISA test, used in the EU, was unsuccessful for pork material heated to 127°C at the core of the sample, i.e. 6°C below the recommended temperature. Furthermore, von Holst et al. (1999) demonstrated that animal meal has to contain at least 10% of pork material to produce a reliable *R*-value threshold. These authors demonstrated that a low *R*-value does not necessarily prove compliance with the legal requirements. Similarly, we found that 22 (66%) of 41 PCR positive samples from Swiss companies (Table 2) gave *R*-values below 3. The suggestion of von Holst et al. to lower the threshold *R*-values from 3 to 2 is justified. Also, the PCR test shows that as little as 0.5% of poorly heat treated animal meal can easily be detected (Fig. 2).

5. Conclusion

From the present results, we can draw the following conclusions: (1) ELISA and PCR tests can both confirm

if the prescribed heat treatment conditions (wet sterilization at 133°C for 20 min at 3 bar pressure) are fulfilled by MBM manufacturers. (2) The ELISA test does not perform well when there are low levels of poorly heat-treated pork-based materials within the sample. (3) The ELISA test is not appropriate to detect adulteration of compound feedingstuffs with non sterilized MBM or meat meals. (4) The PCR assay has limitations, as it will not detect properly heat-treated bovine material in feedingstuffs.

The above conclusions suggest the need for a bovine species-specific PCR test for the specific determination to ensure proper heat treatment procedures have been applied. In this context, we recommend the PCR assay of Matsunaga et al. (1999), which was found in this investigation to be a rapid, sensitive, and specific method for the routine screening of animal meals for the prophylaxis of BSE.

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