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THE DETECTION OF ANIMAL DERIVED DNA IN STOCKFEED USING REAL-TIME PCR

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TABLE OF CONTENTS

ABSTRACT

The practice of feeding meat and bone meal (MBM) to cattle has been banned following its implication in the spread of Bovine spongiform encephalopathy (BSE) through the cattle population. Australia is in a very low risk category for BSE in cattle. However, in order to maintain this status and ensure that the ban on feeding ruminant derived materials to cattle and sheep is implemented, the ability to monitor for the presence of animal-derived tissued in stockfeed is considered critical. A major objective of this study was to develop a test that would detect the presence of vertebrate tissue in stockfeed and, in the first instance, to focus on developing a test that would detect the main animal species of interest, beef and sheep. A test has been developed that detects the presence of either ovine or bovine DNA extracted from rendered meat. The method has been validated for detection of rendered meat from these species in stockfeed and a Reporting Threshold established. A series of stockfeed samples were spiked with either 0.5% rendered bovine or ovine tissue. Based on the Reporting Threshold, more than 95% of these samples would be reported as positive. Samples of rendered chicken and fish were not detected nor did the test cross-react with human DNA.

EXECUTIVE SUMMARY

Bovine spongiform encephalopathy (BSE) is a fatal degenerative disease affecting the central nervous system of cattle. The disease was first reported in the UK in 1986, with up to 100,000 cattle affected by 1993. There are several theories as to the origin of BSE and the mechanism by which it is spread. One theory is that it originated from a spontaneous mutation in a cow but a second theory is that it was transmitted from scrapie infected sheep meat in stockfeed. In the past it has been common practice to produce meat and bone meal (MBM) from the waste meat and skeleton of animal tissues that could not be used for human consumption and to incorporate the MBM into animal stockfeed. The practice of feeding MBM to cattle has been implicated in the lateral transmission of BSE through the cattle population (1). More than 80 cases of a variant of the fatal Creutzfeldt-Jakob disease (nvCJD), which affects humans, have been reported in the UK. This human disease has been linked etiologically to BSE (2).

In 1996, the World Health Organisation made a recommendation that ruminant protein should not be included in cattle feeds. This was implemented as a preventative measure to avoid the spread of BSE among cattle population and to minimize transmission of BSE from bovines to humans. Since 1988, a ban on addition of animal derived materials in cattle feeds has led to a decline in the incidence of BSE in Great Britain (3). With the recent increased incidence of BSE in Europe, a ban on the feeding of all animal-derived material is currently in place in Europe. This ban excludes tallow.

Australia currently has a BR Level 1 rating and is placed at very low risk group for BSE in cattle. However control measures, to ensure that bans on feeding ruminant derived materials to cattle and sheep are implemented, are considered a critical element in the maintenance of this status.

The major objective of this study was to develop a test that would detect the presence of vertebrate tissue in stockfeed and, in the first instance, to focus on developing a test that would detect the main animal species of interest, bovine and ovine. The Australian Government Analytical Laboratories (AGAL) has previously developed a universal test to detect DNA extracted from fresh vertebrate tissue using the polymerase chain reaction (PCR) (4). However, preliminary investigations indicated that this method has limited application for stockfeed where the meat material has undergone a rendering process and the DNA has been significantly degraded.

Following these initial studies, a new assay was designed, specifically, to detect and amplify a much shorter fragment of the cytochrome B (*cytb*) gene. This region of the *cytb* gene is highly conserved among several animal species including bovine, ovine, caprine and porcine. For this reason, it was predicted that such an assay would be suitable for the detection of several vertebrate species. The method was developed and validated for detection of rendered ovine or bovine material in stockfeed.

The specificity of the test for other animal species was examined by analysis of rendered meat from pig, chicken and fish and by analysis of DNA extracted from goat, horse and human tissue. The test showed strong reactivity with DNA extracted from caprine tissue and weak reactivity with rendered pork samples.

Samples of rendered chicken and fish were not detected nor did the test detect DNA extracted from either horse or human tissue. A range of stockfeed samples were spiked with either 0.5% rendered bovine or ovine tissue (n=17). Based on reporting criteria that were established in the validation process, more than 95% of these spiked samples would be reported as positive.

The PCR method developed and described herein can be used as part of the auditing process to monitor compliance with the Australian Government's legislation banning the addition of animal-derived tissue to stockfeed. This will not only assist in the protection of Australia's meat industry from the threat of BSE transmission among the cattle population but will also support access of Australia's beef and mutton to international markets.

Note: The practice of feeding MBM, the cattle feed made from the rendered remains of dead animals, to cattle has been implicated in the spread of BSE through the cattle population. However, a safe level of rendered meat in stockfeed that will exclude transmission of BSE has not been established. Presence of rendered bovine or ovine material in stockfeed below the Reporting Threshold established in this method does not exclude the possibility that BSE could be transmitted via that stockfeed.

BACKGROUND

Bovine spongiform encephalopathy (BSE) is a fatal degenerative disease affecting the central nervous system of cattle. The disease was first reported in the UK in 1986, with up to 100,000 cattle affected by 1993. One theory is that the disease originated from a spontaneous mutation in a cow. The practice of feeding meat and bone meal (MBM), the cattle feed made from the rendered remains of dead animals, to cattle has been implicated in the spread of BSE through the cattle population (1). More than 80 cases of a variant of the fatal Creutzfeldt-Jakob disease (nvCJD), which affects humans, have been reported in the UK. This human disease has been linked etiologically to BSE (2).

In 1996, the World Health Organisation made a recommendation that ruminant protein should not be included in ruminant feeds. This was implemented as a preventative measure to avoid the spread of BSE and to minimize transmission of BSE from bovines to humans. With the recent increased incidence of BSE in Europe, a ban on the feeding of all animal-derived material is currently in place in Europe. This ban excludes tallow.

Australia currently has a BR Level 1 rating and is placed at very low risk group for BSE in cattle. However control measures, to ensure that bans on feeding ruminant derived materials to cattle and sheep are implemented, are considered a critical element in the maintenance of this status.

The PCR method developed and described herein can be used as part of the auditing process to monitor compliance with the Australian Government's legislation banning the addition of animal-derived tissue to stockfeed. This will not only assist in the protection of Australia's meat industry from the threat of BSE transmission but will also support access of Australia's beef and mutton to international markets

OBJECTIVES

The major objective of this study was to develop a test that would detect the presence of vertebrate tissue in stockfeed and, in the first instance, to focus on developing a test that would detect the main animal species of interest, beef and sheep.

METHODOLOGY

The PCR methodology developed by AGAL for this analysis is based on targeting a known sequence within a genome and the production of many copies of this sequence via an amplification process. The specific sequence targeted is a conservative region of the cytochrome B (*cytb*) gene that is present in all animal species but not in plants or bacteria. The *cytb* gene is a mitochondrial gene present in large copy numbers, resulting in a highly sensitive assay.

It was considered important to develop an assay that would detect animal tissue in stockfeed not only from bovine but also from other closely related ruminant species such as ovine because of the possibility that sheep infected with scrapie could also be a mechanism for transmission of BSE. Although the evidence of the transmission of BSE by other closely related ruminant species such as buffalo and goat has not yet been identified, several countries including Australia have banned the addition of any animal derived materials into stockfeed in order to minimise the risk of BSE transmission through stockfeed.

Four main criteria were considered in the development of the test. The first requirement was that the assay target a region of the genome that is highly conserved among several animal species. The *cytb* gene is conserved across several species including bovine, ovine, caprine and porcine. For this reason, it was predicted that such an assay would be suitable for the detection of several vertebrate species. The second criterion was that the targeted sequence should be around 100 base pairs in length. This criterion was based on previous studies at AGAL and by other workers that demonstrated a reduced sensitivity of tests that targeted a larger sequence (>250 base pairs) when amplifying DNA extracted from rendered meat as compared to fresh or frozen tissue (5, 6). It is well known that genomic DNA is degraded to fragments of approximately 100 to 200 bp during the severe, prolonged conditions of high temperature (130°C) and pressure (3 pa) achieved during rendering. The third criterion was that the targeted region of DNA be relatively stable so that it would not undergo significant degradation during the rendering process. The fourth criterion related to the potential for cross-reactivity with human DNA since such contamination is one of the major concerns for a diagnostic PCR designed to identify closely related animal species. Hence the assay was intentionally designed to identify the main animal species of interest without exhibiting any cross-reactivity with human DNA.

The method described involves the steps of sample homogenisation, DNA extraction (Wizard Genomic DNA Purification Kit, Promega) and DNA purification (QIAquick® PCR column purification kit, Qiagen), followed by amplification of the targeted DNA sequence using real-time PCR (ABI 7700 Sequence Detection System). Sybr Green, a dye that fluoresces when bound to double-stranded DNA, is present in the reaction mix and the amount of PCR product in the sample is determined by measuring the level of fluorescence accumulation in real time. When the fluorescence reaches a level associated with exponential growth of the PCR product, this is referred to as the Threshold Cycle (C_T). The C_T value is calculated for each sample and is dependent on the amount of target DNA in the initial sample. Hence, a sample with a large amount of target DNA would require fewer cycles to reach the C_T than a sample with a very small amount of target DNA. When no amplification has occurred, reporter fluorescence is not significantly different to the baseline signal level.

A minimum of 10 gram of stockfeed pellets is required for analysis. DNA extraction is performed in duplicate for each sample and then each extract amplified by PCR reaction. If the PCR amplification is to be commenced within 2 hours of DNA extraction, the DNA is stored at 4^{o}C otherwise DNA is stored at –20^{o}C.The PCR should be performed with 24 hours of DNA extraction. The PCR conditions include a hot start (95°C for 10 minutes) followed by 40 cycles of denaturation (95°C for 15 seconds) and annealing/extension (60°C for 1 minute). Rendered bovine material and control samples of plant-based stockfeed and 0.5% rendered bovine/ovine in plant-based stockfeed must be included with each assay. A ‘No template’ control (DNA is replaced by distilled water) is also included in each PCR analysis.

RESULTS AND DISCUSSION

Preliminary analysis of plant-based stockfeed and rendered meat

Several plant-based stockfeeds were obtained from commercial stockfeed manufacturers. In addition, samples of rendered bovine, ovine and porcine meat and rendered chicken and fish samples were obtained from commercial rendering plants. Initial studies indicated that the PCR test would detect rendered ovine and bovine material, and that the C_T value for plant-based stockfeed spiked with 0.5% rendered ovine material was significantly lower than the C_T value for plant-based stockfeed. A series of experiments were then conducted to validate the assay and to establish performance and reporting criteria.

Specificity and Sensitivity of the method

The specificity of the method was determined by analysis of a range of animal- and plant-based materials. Genomic DNA was analysed from several animal species including bovine, ovine, porcine, caprine, equine, human, chicken and fish. The assay detected DNA from bovine, ovine, goat and porcine but not from horse, human, chicken or fish (Table 1). The assay showed strong reactivity to ovine, bovine and goat DNA but was less sensitive in the detection of porcine DNA. The lack of cross-reactivity with human DNA indicated that human DNA contamination would not interfere with the analysis.

Method validation concentrated on the major species of interest, bovine and ovine tissue. The sensitivity of the method was defined by analysis of plant-based stockfeeds spiked with rendered meats. Plant-based stockfeed was spiked with 0.1%, 0.2%, and 0.5% (w/w) rendered bovine or ovine meats, respectively. Total genomic DNA was then extracted and analysed to determine the sensitivity of the assay. There was a significant difference in the C_T values for plant-based stockfeed samples in comparison to the same stockfeed spiked with 0.5% rendered meat (Tables 1 and 2).

Several agarose gel based PCR methods have been developed previously for species identification in food and feedstuffs (3, 7-9). One method is based on amplification of a 271 bp fragment of the bovine ATPase8 mitochondrial gene and reportedly can detect down to 0.125% bovine material in food and stockfeed. (3, 7, 8). Since this gene is less conserved among vertebrates than the cytochrome B gene, this method did not detect DNA from other closely related ruminant species. In contrast, AGAL focused on development of a real-time PCR method that would detect a short 125 bp DNA fragment in the conserved *cytb* gene.

Analysis of blind stockfeed samples of known composition

Thirty-five commercially prepared stockfeed samples of known composition were supplied to AGAL for analysis. As part of the validation process the samples were analysed blind and their composition was only revealed to the laboratory after completion of analysis. The results demonstrated that the method is both accurate and sensitive and can detect animal derived DNA extracted from a complicated matrix such as commercially prepared stockfeed. No false negative and false positive results were obtained from this blind study (Table 3). The blind samples used in this analysis were commercial available stockfeeds, and, when present, the concentration of animal tissue ranged from 4.75 to 10%. This is much higher than the 0.5% test limit of the assay. Although commercial stockfeeds containing very low levels of animal material were not available for analysis, the sensitivity study using spiked samples demonstrated that the assay can detect down to 0.5% rendered meat in plant-based stockfeeds.

Establishing performance criteria

Performance criteria were established to monitor the analysis of sample batches. The repeatability of the assay was determined on 3 samples; a plant-based stockfeed (SFB), SFB spiked with 0.5% rendered ovine material and SFB spiked with 0.5% rendered bovine material. Repeatability was determined by comparison of C_T values for repeated analysis (n = 7) of the same sample on the same day by the same operator. Based on these results, criteria were established for the repeatability of the C_T value in duplicate analyses of samples and controls.

Reproducibility of the assay was determined by analysis of 2 samples; SFB and SFB spiked with 0.5% rendered ovine material. Reproducibility was determined by repeated analysis (n = 7) of the sample on different days using at least 2 different operators. Based on these results, upper and lower control limits were established for the C_T value for SFB and for SFB spiked with 0.5% rendered ovine.

Establishing reporting criteria

Criteria were established for reporting samples below the Limit of Detection and for reporting samples as positive. A sample would be considered as below the Limit of Detection if the difference between the total number of PCR cycles and the C_T value for the sample is less than 3 times the standard deviation of the C_T value as estimated from the repeatability analysis.

A Reporting Threshold was established such that samples with a C_T value that falls below the Reporting Threshold are reported as positive. The Reporting Threshold was established based on analysis of a range of plant-based stockfeed samples spiked with either 0.5% rendered ovine or bovine material and was set so that the probability of a stockfeed sample spiked with 0.5% rendered meat falling below the Reporting Threshold was 95% (Table 3).

CONCLUSIONS

A real-time PCR assay has been developed and validated for the detection of DNA extracted from either rendered bovine or ovine tissue present in stockfeed. The method targets a short DNA fragment within the cytochrome B gene which appears to be relatively stable to the harsh conditions of high temperature and pressure that are achieved and maintained during the rendering process.

This method can be used by regulatory agencies as an auditing tool to monitor the contents of feedstuffs and to ensure compliance with the Australian Government's legislation banning the addition of bovine and ovine tissue into animal feed. Therefore, the method should be a significant contribution to the maintenance of Australia's BR Level 1 rating. This will not only assist in the protection of Australia's meat industry from the threat of BSE transmission to human but will also support access of Australia's beef and mutton to international markets.

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APPENDICES

Table 1: Analysis of the specificity of the stockfeed test

Sample Type	Sample	Source	C_T value		Reported as[a]
			1:10 dilution	1:50 dilution	
Non Template Control					
	Water	Microbiology,AGAL	39.47		<LOD
Vertebrate-fresh or frozen meat[b]					
	Human	Lab. Staff, R&D	39.60		<LOD
	Pig	R&D, AGAL	24.44		-
	Cattle	R&D, AGAL	19.98		POS***
	Goat	R&D, AGAL	20.06		POS***
	Horse	R&D, AGAL	40		<LOD
	Bovine indicus	New England University	17.92		POS***
	Bovine taurus	New England University	18.48		POS***
Vertebrate-rendered					
	Ovine7-10	Fletcher International Exports	15.75		POS***
	Bovine11-12	Wingham Beef Exports	17.06		POS***
	Porcine14	Camilleri Stockfeeds P/L	26.44		POS***
	Fish15-16	Camilleri Stockfeeds P/L	35.83		-
	Chicken1-5	Baiada Poultry	36.56		<LOD
Powdered Milk Products					
	100% Milk powder	Dutch Jug	31.07	33.55	-
	10% Milk powder in SFB	Dutch Jug	31.02	31.19	-
	10% Milk powder, 0.5% rendered bovine in SFB	Dutch Jug	26.92	29.21	POS***
Plant based stockfeeds[b]					
	SFA	Weston Animal Nutrition	30.40	33.02	-
	SFB	Weston Animal Nutrition	28.57	31.29	-
	SFC	Weston Animal Nutrition	31.77	33.87	-
	Brendans	AustAsia	36.86	37.84	<LOD
	Lampong	AustAsia	35.25	38.96	-
	Shipping	AustAsia	32.93	35.69	-
	Phillipine	AustAsia	36.24	37.385	<LOD
	Finisher	AustAsia	36.57	36.285	<LOD
Plant Raw Materials					
	Soy powder Certified Reference Material	IRMM, Belgium	38.12	40	<LOD
	Maize flour Certified Reference Material	IRMM, Belgium	36.67	39.04	<LOD

[a] <LOD refers to C_T value less than the Method Detection Limit; POS*** refers to C_T value below the Reporting Threshold hence sample reported as Positive.

[b] The final concentration of DNA extracted from these samples was 10-15 ng/μl.

Table 2: Analysis of stockfeed samples spiked with 0.5% (w/w) rendered meat

Date	Rendered Sample	Stockfeed	Ct values		Reported as
			1:10 dilution	1:50 dilution	
3/11/01	0.5% bovine	SFB	27.46	29.07	POS***
26/09/01	0.5% ovine	SFB	25.96	27.81	POS***
19/09/01	0.5%ovine/bovine	SFC	25.60	27.95	POS***
3/11/01	0.5% bovine	Brendans	24.78	27.00	POS***
3/11/01	0.5% bovine	Lampong	25.68	27.94	POS***
3/11/01	0.5% bovine	Shipping	25.47	28.28	POS***
3/11/01	0.5% bovine	Finisher	23.49		POS***
3/11/01	0.5% bovine	Phillipines	24.95	26.92	POS***

Mean (x)	25.42	27.85
Reproducibility SD (σ_R)	1.13	0.74
Reporting Threshold = X+ 2σ	27.7	29.3

Table 3: Analysis of blind stockfeed samples

Sample Number	C_T value		Reported as[a]	Sample Code	Feed Type[b]	Meat Meal (%)[c]	Blood Meal (%)[d]	Poultry Meal (%)
	1:10 dilution	1:50 dilution						
BS1	33.74	36.80	-	683-03	Dairy Cattle	0	0	0
BS2	31.44	34.52	-	582-08	"	0	0	0
BS3	29.90	32.17	-	682-77	"	0	0	0
BS4	32.12	34.45	-	680-61	"	0	0	0
BS5	25.24	26.91	POS***	117-49	Broiler Chicken	5.5	0.5	0
BS6	22.88	25.81	POS***	137-80	"	5	0	2.5
BS7	30.10	32.11	-	594-19	Dairy Cattle	0	0	0
BS8	23.90	26.24	POS***	128-50	Broiler Chicken	5.75	1.25	0
BS9	24.19	27.09	POS***	123-42	"	7.75	0.5	0
BS10	22.58	25.34	POS***	133-51	"	8.5	0.5	0
BS11	28.41	31.43	-	590-16	Dairy Cattle	0	0	0
BS12	28.57	30.67	-	645-55	"	0	0	0
BS13	22.08	24.53	POS***	121-66	Broiler Chicken	5.75	0.75	0
BS14	29.37	32.15	-	736-16	Dairy Cattle	0	0	0
BS15	PCF[e]	25.71	POS***	147-36	Broiler Chicken	7.75	0	0
BS19	22.27	PCF	POS***	103-52	"	10	0	0
BS20	31.33	33.72	-	643-03	Dairy Cattle	0	0	0
BS22	22.31	25.91	POS***	128-61	Broiler Chicken	5.75	1	0
BS24	24.79	27.97	POS***	127-91	"	5.75	0	2.75
BS25	31.86	33.91	-	592-14	Dairy Cattle	0	0	0
BS27	23.45	26.51	POS***	137-01	Broiler Chicken	8	0	2.5
BS28	21.70	23.53	POS***	121-66	"	5.75	0.75	0
BS29	32.61	35.22	-	594-19	Dairy Cattle	0	0	0
BS30	23.39	26.37	POS***	127-78	Broiler Chicken	5.75	0	2.5
BS31	28.86	30.52	-	680-61	Dairy Cattle	0	0	0
BS32	30.24	33.22	-	736-16	"	0	0	0
BS33	PCF	35.89	-	730-03	"	0	0	0
BS34	24.43	27.59	POS***	137-62	Broiler Chicken	4.5	0.75	4.5
BS35	23.76	26.70	POS***	147-74	"	4.75	0	2.75
BS19	22.26	24.56	POS***	103-52	"	10	0	0

[a] POS*** refers to C_T value below the Reporting Threshold hence sample reported as Positive.

[b] There was no milk product in any of the rations and no tallow in the bovine feed. The broiler feed rations contained tallow at the level of 0.5-3%.

[c] The meat meal was believed to contain bovine and/or ovine material and possibly porcine material. However, the precise composition was unknown.

[d] The precise composition of the blood meal was unknown.

[e] PCF; Performance criteria for repeatability of duplicates not met at this dilution

Definitions

AGAL	Australian Government Analytical Laboratories
bp	base pairs
BSE	Bovine spongiform encephalopathy
C_T	threshold cycle
DNA	deoxyribonucleic acid
MBM	Meat and bone meal
nvCJD	variant Creutzfeldt-Jakob disease
PCR	polymerase chain reaction