CHAPTER 2C

Ruminant DNA detection: Real-time PCR Detection of Ruminant DNA

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Abstract

In order to control the spread of transmissible spongiform encephalopathy (TSE), several DNA methods have been described for the detection of the species origin of meat-and-bone-meal. Most of these methods are based on the amplification of a mitochondrial DNA segment. We have developed a semi-quantitative method based on real-time PCR for detection of ruminant DNA, targeting an 88-bp segment of the ruminant short interspersed nuclear element (SINE) Bov-A2. This method is specific for ruminants and is able to detect as little as 10 fg of bovine DNA. Autoclaving decreased the amount of detectable DNA, but positive signals were observed in feedstuff containing 10% bovine material if this had not been rendered in accordance with the regulations, i.e. heated at 134°C for 3 instead of 20 minutes.

1. Introduction

The use of meat and bone meals (MBM) from sheep in feedstuff for cattle has been implicated in the emergence of BSE (bovine spongiform encephalopathy) (Taylor and Woodgate 1997). Human consumption of beef from BSE-infected cattle has caused the emergence of a juvenile form of Creutzfeldt-Jakob's disease (CJD) in man, the new variant CJD (vCJD) (Will 1999). After the 1988 UK ban on feeding ruminant material to other ruminants, European guidelines now prohibit with a few exceptions the addition of any processed animal material to feedstuff unless the absence of ruminant proteins can be demonstrated (EEC Commission decision 94/381/EEC). In order to enforce this, several tests have been developed to detect animal material in feedstuff. Currently, the official method in the European Union is the detection of animal bone fragments by microscopy. This method, however, cannot distinguish bone fragments in MBM if these are finely grinded. Furthermore, this method does not allow identification of the species and requires trained personnel. Commercially available ELISA kits detect species-specific antigens, but are only partially adequate for samples that have been subjected to a heat treatment (Von Holst et al. 2000).

Because of its heat stability, DNA is a suitable target for species identification assays. Dot-spot hybridization is suitable for detection of species-specific DNA repeats in

heated or even autoclaved meat samples (Buntjer *et al.* 1999; Buntjer *et al.* 1995), but does not have the sensitivity required for analysis of feedstuff (unpublished results). PCR-based methods are more sensitive and several protocols are available for the extraction and analysis of DNA from feedstuff (Table 1). Most of these are based on the detection of species-specific mitochondrial DNA sequences and determine only the presence of bovine material (Kingombe *et al.* 2001; Krcmar and Rencova 2001; Lahiff *et al.* 2002; Tartaglia *et al.* 1998), use generic primers (Kocher *et al.* 1989; Meyer and Candrian 1996) that do not perform equally well in all species (Verkaar *et al.* 2001) or yield only qualitative results. Two recently developed methods (Tajima *et al.* 2002; Walker *et al.* 2003) are targeted to SINE repetitive elements specific for the genomes of ruminants, pigs and poultry, respectively.

The amplification of these SINE sequences and of a bovine-specific satellite DNA sequence has been combined with real-time detection by measuring SYBR Green fluorescence (Walker *et al.* 2003). However, SINE elements are heterogeneous (Lenstra *et al.* 1993) and the design of PCR primers that match a significant proportion of the SINE copies may not be straightforward.

Detection of ruminant material in the food chain is especially relevant because of the prevalence of transmissible spongiform encephalopathy in at least three ruminant species and the infectivity of abnormal bovine prions in men (Dalton and Check 2002; Hamir *et al.* 2001; Taylor and Woodgate 1997; Will 1999). As a further refinement of a quantitative and specific detection of ruminant DNA, we describe here a 5'-nuclease or Taqman® assay based on the relatively homogeneous Bov-A2 SINE element (Lenstra *et al.* 1993). With this method, real-time fluorescent detection is generated by the hydrolysis of a third, internal oligonucleotide, which increases the specificity relative to a normal two-primer PCR. An alignment of Bov-A2 elements allowed the selection of the primers and the Taqman probe in the most conserved region of the SINE sequence, while the sensitivity is further optimized by reducing the size of the amplicon to 88 bp. The sensitivity and specificity of this method as well as results with experimental MBM and feedstuff samples is described.

2. Materials and methods

2.1 Samples and DNA extraction

Genomic DNA was isolated from blood samples by using the Qiagen blood extraction kit (Qiagen Inc., Valencia-CA, USA) or by GuITC (guanidium-isothiocyanate) extraction (Ciulla *et al.* 1988; Sambrook *et al.* 1989)**.** DNA concentrations were determined spectrophotometrically. Experimental MBM samples provided by the Institute of Animal Science and Health (ID-DLO, Lelystad) contained 90% predominantly porcine material and 10% bovine brain material. The samples have been exposed to various heat treatment cycles performed in an autoclave with internal monitoring conditions. Feedstuff samples prepared by Labocor (Madrid) contained equal amounts of maize, soya, barley and wheat with commercial MBM at quantities ranging from 0.1 to 10 %. DNA from 200 mg of feedstuff was extracted with the Wizard Magnetic DNA purification system for food (PROMEGA, Madison WI, USA).

2.2 Quantitative PCR of the Bov-A2 segment

Primers and an internal probe specific for the ruminant Bov-A2 SINE consensus sequence Genbank entry X64126 (Lenstra *et al.* 1993) were designed using the Primer Express program (Applied Biosystems, Foster City-CA, USA). Primers (5'-GAC TGA GCG ACT TCA CTT TCA) and (5'-GGA TTC TCC AGG CAA GAA CA) amplify a fragment of 88 bp (position 104—191 in Genbank entry X64126). The 5'-nuclease Taqman® probe (5'-FAM-5'-TTG GAG AAG GAA ATG GCA ACC CAC TCC-TAMRA-3') was synthesized by Eurogentec (Seraing, Belgium). Real-time PCR has been performed in a total volume of 25 µl, containing 12.5 µl Taqman Mastermix (Applied Biosystems), 0.45 mM of both primers and 0.2 mM probe. The program included 10 min predenaturation at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. PCR amplification and detection was performed on a Taqman 7000 instrument (Applied Biosystems).

Table 1. DNA based assays of feedstuff

Reference	Target	Amplicon length	Detection method
Krcmar and Rencova 2001; Myer et al. 2001; Tartaglia et al. 1998; Wang et al. 2000	Bovine ATPase subunits 6 and 8	271	PCR, electrophoresis
Frezza et al., 2003	Bovine ATPase subunits 6 and 8	271, 240, 147	Competitive PCR, electrophoresis
Lahiff et al., 2002	Bovine ATPase 8 subunit	271	PCR, online 5' nuclease assay
Bellagamba et al., 2001	Vertebrate mitochondrial cytochrome b	359	PCR-RFLP
Bellagamba et al., 2003	Ruminant mitochondrial 12 S rRNA gene	231	PCR, agarose gel electrophoresis
	Pig mitochondrial 12 S rRNA gene	186	
	Poultry mitochondrial 12 S rRNA gene	256	
Kingombe et al. 2001	Vertebrate mt $tRNA^{Glu}$-cytochrome b	464	PCR, electrophoresis
	bovine mtDNA	274	"
Colgan et al. 2001; Lahiff et al. 2001	Bovine ATPase 8 subunit	271	PCR, electrophoresis
	Ovine ATPase 8 subunit	225	
	Porcine ATPase 8 subunit	212	"
	Chicken ATPase 8 subunit	266	"
Tajima et al. 2002	Ruminant Bov-B SINE	181	PCR, electrophoresis
	Porcine PRE-1 SINE	179	"
	Avian CR-1 SINE	201	"
Walker et al. 2003	Bovine satellite 1.711B	98	PCR, online SYBR green-fluorescence
	Porcine PRE-1 SINE	134	
	Avian CR1 SINE	169	
	Ruminant Bov-tA SINE	100	
This paper	Ruminant Bov-A2 SINE	88	PCR, online 5' nuclease assay

3. Results

For the development of a ruminant-specific quantitative PCR assay the Bov-A2 SINE repeat was chosen as target because it is more homogeneous than other major SINE sequences Bov-tA and Bov-B (Lenstra *et al.* 1993). Primers and probe were designed by following the guidelines of Applied Biosystems in Primer Express and were located in the most conserved part of Bov-A2. A BLAST search showed that the forward primer, the reverse primer and the Taqman probe had 54, 97 and 97 perfect matches in 13.4 Mb of bovine DNA and 5, 14 and 9 perfect matches in 1.9 Mb of ovine DNA, respectively. The optimal concentrations were found to be 0.45 mM for both primers and 0.2 mM for the probe.

Fig. 1 shows the analysis of tenfold dilutions of bovine genomic DNA. Although the Ct value is less accurate at the lowest DNA concentration, 8.5 fg bovine DNA still gave signals significantly higher than the blank value. Plots of Ct values versus the logarithm of the DNA concentration (Fig. 2) show good linearity ($R^2 = 0.995$). Essentially the same correlation between DNA concentrations and Ct values was obtained with sheep and goat DNA, respectively. DNA from other ruminants (giraffe, mule deer) also generated clearly positive signals (Table 2). With DNA from various non-ruminant species, Ct values were 25 or higher (Table 2) and probably correspond to traces of bovine DNA in the sample.

The scrapie-agent is capable of withstanding temperatures of up to 137°C (Brown *et al.* 2003). We investigated detection limits of bovine DNA in samples of feedstuff mixed with 10% bovine brain tissue subjected to different heat treatments (Table 3). Bovine DNA could still be detected after 3 min at 134°C, but the amount of DNA decreased by a longer heat treatment (20 min at 133°C) or by heating at a higher temperature (3 min at 140°C). However, even after treatment under these extreme conditions 5 to 18 pg DNA could still be detected. In commercial feedstuff samples containing MBM only 4 to 70 fg ruminant DNA was measured (data not shown), which is around the detection limit.

Table 2. Specificity of the BovA detection

Species	Amount of DNA (ng)	Ct value
Mule deer	125	11.5
Giraffe	40	13.1
Pig	12.5	27.7
Horse	12.5	29.2
Chicken	12.5	31.0
Turkey	12.5	32.1

Table 3. Ruminant DNA detected in 200 mg feed stuff samples containing 10% bovine brain material and subjected to the indicated heat treatment. Amounts of DNA were read from a calibration curve as in Figure 1 based on the analysis of 850 pg to 85 fg bovine genomic DNA (Ct 14.1 to 27.3, 31.2 for blank sample) in the same run

Heat treatment	DNA (pg)
100°C, 20 min	1082
105°C, 3 min	255
121°C, 3 min	777
121°C, 3 min	255
121°C, 15 min	527
134°C, 3 min	270
134°C, 3 min	249
133°C, 20 min	5.2
133°C, 20 min	1.7
140°C, 3 min	17.6
140°C, 3 min	5.0

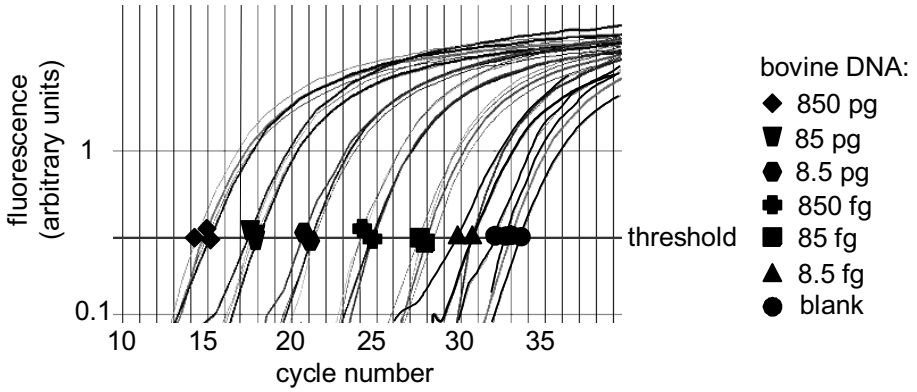

Figure 1. Fluorescent profiles of the PCR reaction with tenfold dilutions of genomic bovine DNA and a blank

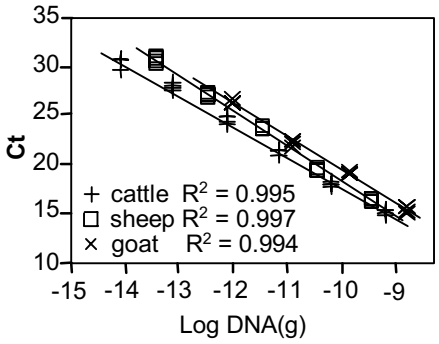

Figure 2. Ct values (cycle number at which the fluorescence passes the threshold, see Fig 1) *vs* the logarithm of the amount of bovine genomic DNA (g) and corresponding regression data

4. Discussion

Growing evidence for causal relations of BSE in cattle and vCJD (Will 1999) has led to strict regulations on the composition of feedstuff and a requirement of test methods in order to be able to enforce the rules (Taylor and Woodgate 1997). Current EU legislation prohibits with a few exceptions the addition of any animal material. Most assays of feedstuff described so far (Table 1) test only for bovine material and will not detect material from other ruminants like sheep and deer, which are potentially infected with scrapie or chronic wasting disease (CWD), respectively (Dalton and Check 2002; Hamir *et al.* 2001).

Ideally, a test should have a well-defined and relevant species-specificity, a linear response, an adequate sensitivity for degraded DNA, and a high throughput.

We have developed a real-time PCR assay for the presence of the ruminant-specific Bov-A2 SINE, which is estimated to account for 1.8% of the bovine genome (Lenstra *et al.* 1993). SINE elements have a broader taxonomic range than the species-specific centromeric satellite repeats (Jobse *et al.* 1995). As a consequence, SINE sequences are heterogeneous and primers may match to only few genomic SINE copies. For instance, one of the primers designed for the amplification of the more degenerate Bov-tA elements (Walker *et al.* 2003) has only few complete matches to bovine or ovine SINE sequences in the nucleotide database and the second primer has no complete match at all. The Bov-A2 SINE is more homogeneous than the related Bov-tA and Bov-B elements (Lenstra *et al.* 1993) and is present in comparable amounts in the genomes of all ruminants (Buntjer *et al.* 1997; Jobse *et al.* 1995; Nijman *et al.* 2002). Our primers have been optimized for cattle, but detect also DNA from sheep, goat, deer and giraffe.

The specificity of our test has further been enhanced relative to gel electrophoresis or SYBR-Green detection (see Table 1) by the use of a third target-specific oligonucleotide in a 5'-nuclease detection. This may be essential for critical samples and also allows a multicolor multiplex detection (Walker *et al.* 2003). The FAM-TAMRA probe used in this study matches a conserved region and has several matches to ruminant SINE elements in the nucleotide database.

With cattle DNA the signal was found to be linear over a million fold range of DNA concentrations with essentially the same response with sheep and goat DNA, respectively.

The detection limit of 10 fg compares favorably with the detection of 1 pg achieved by competitive PCR (Frezza *et al.* 2003) or of 0.1 pg achieved by on-line PCR of the Bov-tA SINE with SYBR detection (Walker *et al.* 2003) and is in the same range as the 18.75 fg reported for a Bov-B SINE PCR (Tajima *et al.* 2002). An important factor that in practice may limit the sensitivity is contamination with bovine DNA, for instance during sample processing or by the frequent use of bovine serum albumin in biochemical reagents.

The sensitivity for degraded DNA samples was optimized by a short amplicon length. Analysis of a panel of experimental MBM samples containing 10% bovine brain material indicated that bovine DNA can be detected even after heating at 134°C for 3 min. The signal of DNA detected after 20 min at 133°C or 3 min at 140°C corresponds to 5 to 18 pg DNA. These values probably indicate a contamination of the experimental MBM samples after the heat treatment, since lower values were measured in commercial MBM or in the blank sample.

An attractive feature of the on-line detection is that no post-PCR steps are required and that the method is suitable for automated high-throughput analysis. Although a further validation with experimental and practical feedingstuff samples is required, there is proof-of-concept of the quantitative assay of the Bov-A2 SINE or other repetitive elements (Tajima *et al.* 2002; Walker *et al.* 2003) as tools for the detection of the origin of feedstuff and for the control of the spread of transmissible encephalopathic diseases.

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