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ScienceDirect
Innovative Food Science and Emerging Technologies 9 (2008) 18–23
Innovative Food Science & Emerging Technologies
www.elsevier.com/locate/ifset

Standard and Light-Cycler PCR methods for animal DNA species detection in animal feedstuffs

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Received 15 August 2006; accepted 14 April 2007

Abstract

In this work four species-specific primers and probes were designed and evaluated for the detection and quantification of bovine, ovine, swine and chicken mitochondrial DNA in feeds. PCR primers were optimized using conventional and Real Time PCR, to detect short species-specific sequences amplifiable from heat treated material. Both methods confirmed the high specificity of the primers designed. Real time quantitative PCR assay allowed the detection of as few as 0.01 ng and 0.05 ng of ovine and bovine genomic DNA, respectively. The detection limit for swine and chicken genomic DNA was 0.5 ng. Sensitivity levels observed in DNA extracted from meat samples processed according to EU legislation were different compared to those in genomic DNAs previously described. They resulted in swine 5 fg of MBM DNA, in chicken 25 ng, in ovine and bovine 50 ng. We confirmed the efficiency and specificity of primers in RT-PCR to detect 0.5% of bovine, ovine, swine and chicken MBM in contaminated feedstuffs.
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Keywords: BSE prophylaxis; Rendering material; Species-specific primers; Quantitative real time PCR

Industrial relevance: The variant Creutzfeldt Jakob disease is a rare and fatal human neurodegenerative condition clearly linked with the bovine spongiform encephalopathies (BSE) of cattle. The ban of using animal derived protein in animal feeds has efficiently controlled the development of the BSE epidemic. The work presented by Frezza and collaborators is an application of the real time polymerase chain reaction (a standard procedure used in molecular biology also known as RT-PCR) to identify specific DNA of four animal species (bovine, ovine, swine and chicken). This method is applied to the analysis of feeds to detect and eventually estimate the amount of animal derived proteins. The difficult aim to detect DNA derived from heat-treated material was successfully reached using as target short mitochondrial DNA sequences. The method presented could have important application not only in the control of feed production but also in many fields of the food industry as quality and process control.

1. Introduction

Bovine Spongiform Encephalopathy (BSE) was spread through animal feed containing infected meat and bone meal (MBM) (BSE Inquiry, 2000a, vol 1 and BSE Inquiry, 2000b vol 2). The BSE epidemic first recognized in the UK in 1986 reached most of the European countries. To date 187,000 case of

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doi:10.1016/j.ifset.2007.04.008

the disease have been reported in cattle with more than 98% in the UK (http://www.oie.int). Immediately, procedures had to be implemented to avoid the risk of contamination with infected material and intraspecies recycling. Accordingly, the European Union (EU) introduced restrictions in the production and use of MBM for feeding ruminants and other species of farm animals (Commission Decision 94/381/EC of 27 June, 1994; Commission Decision 94/382/EC of 27 June, 1994; EC (2002) Regulation 1774/2002). Since then, a considerable research effort has been directed towards the development of simple, reliable, high sensibility and low-cost analytical methods to detect MBM in animal feed, to avoid fraud and accidental contamination (see Gizzi et al., 2003 for a review). The first PCR application in MBM detection was reported by Tartaglia et al. (1998). Thereafter, a number of investigations have demonstrated the utility of this technology in the detection and identification of species-specific components in MBM and feedstuffs (Krcmar & Rencova, 2001; Momcilovic & Rasooly, 2000; Myers et al., 2006; Yancy, Mohla, Farrell, & Myers, 2005). PCR methods in fact reach precision and sensitivity levels difficult to achieve using microscopic examination, or impossible applying immuno-enzymatic assays, because of protein instability in heat-treated material (Ansfield, Reaney, & Jackman, 2000; Baeten et al., 2005; Kim et al., 2005). Attempts to establish multiplex PCR (Dalmasso et al., 2004) and real time PCR (RT-PCR) protocols in contaminated food and feedstuff have recently been reported (Krcmar and Rencova, 2005; Lopez-Andreo, Lugo, Garrido-Pertiera, Prieto, & Puyet, 2005; Mendoza-Romero et al., 2004; Taurai, Schumacher, & Roger, 2005). Compared to conventional PCR, RT-PCR has the advantage of quantifying the small size products (from 66 to 145 bp), amplified from highly degraded source material, like rendered MBM. Recently (Frezza et al., 2003), we demonstrated that short amplicons can be used to detect the presence of bovine mitochondrial DNA (mtDNA) in MBM samples treated according to the current European legislation (133 °C, 20′, 3 bars) (Dept. of Agr., Food and Rur. Develop. http://www.irlgov.ie/daff/bse/bse.htm). While potentially offering a very sensitive method for the quantification of specific DNA targets, RT-PCR can produce biased results if applied to DNA concentrations outside the linear range of detection that varies from material to material and amplicon to amplicon. In this paper, we tested the performance of Light-Cycler RT-PCR using species-specific primers and probes to detect and quantify in feedstuffs bovine, ovine, swine and chicken MBM treated according to EU legislation.

2. Materials and methods

2.1. Samples preparation

To prepare MBM from different species, bovine, ovine, swine and chicken meat was chopped at a maximum particle size of 50 mm, autoclaved at 133 °C for 20′ at a pressure of 3 bar (von Holst, Unglaub, & Anklam, 2001) and lyophilised. Commercial feedstuffs samples were contaminated by mixing bovine, ovine, swine and chicken MBM to 100 g of ground material, at final percentage of 0.2%, 0.5% and 1% MBM.

Genomic DNA of samples isolated from tissue and blood from different species, *Bos taurus*, *Sus scrofa*, *Gallus gallus*, *Ovis aries*, was used as positive control. Genomic DNA from mouse, dog, goat, buffalo, deer, horse and wild boar was used as negative control.

2.2. DNA extraction

Total DNA was extracted from 200 mg of meat, MBM and MBM contaminated feedstuffs using Wyzard Magnetic DNA purification system for food (Promega, California, USA), according to manufacturer instructions. DNA concentration was measured by spectrophotometric analysis (Biophotomer,Eppendorf, Germany).

2.3. Primer and probe design

Species-specific primers were designed for the detection of bovine, ovine, swine and chicken DNA (Table 1). Sequences of different mtDNA genes were extracted from GenBank database (http://www.ncbi.nlm.nih.gov) and aligned using Clustal W software (http://www.ch.embnet.org/software/ClustalW.html). Regions showing a low level of intra-species polymorphism and a relatively high degree of divergence among the species investigated were selected to design species-specific primer pairs, amplifying products shorter than 120 bp. To be used in RT-PCR, forward primers in *B. taurus*, *S. scrofa*, *G. gallus* and

Table 1
Species-specific primer, LC probe sequences and amplification conditions

Specie	Primers (5′-3′)	Probes (5′-3′)	Annealing temperature	Target	Size (bp)
Bos taurus	f-CTTGAACTAGACCTAGCCCAAAGATAC r-ATAGCGCCGTACTTAGATTTCTATCTCC	GTTTTATTCTATCTTGGTTGTTTAGTCGAAG	58°C/6 s	16S rRNA	112
Sus scrofa	f-ATGACCAACATCCGAAAATCAC r-TGCCTAAGAGGGAACCGAAG	GCGTTGTTGATAATTTTTATTAGTGGG	52°C/8 s	Cytocrhom b	114
Ovis aries	f-CTTGAACTATACCTAGCCCAAAAT r-ATAGCGCCGTGTTTAGAATTTCTATC	AATTAAAATAAAACATTTACCCTAATTAAAGTATAGG	57°C/8 s	16S rRNA	104
Gallus gallus	f-AGAGCTCGGCAAATGCAAA r-GGCAGGGTCATGTCCG	GAACCTCTGGATAAAGGGCTTAAGCC	50°C/8 s	ATPase 8	99
Plant	f-TGCAGTTAAAAAGCTCGTAG r-GAAACTTCTTTAATCTCACG	CCCGAAGCCGGACCTAGAAAC	50°C/6 s	18S rRNA (*Zea mays*)	159

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