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ScienceDirect
Meat Science 75 (2007) 120–127
MEAT SCIENCE
www.elsevier.com/locate/meatsci

Species-specific PCR for the identification of ruminant species in feedstuffs

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Received 1 January 2006; received in revised form 19 June 2006; accepted 19 June 2006

Abstract

A polymerase chain reaction (PCR) method based on the nucleotide sequence variation in the 12S ribosomal RNA mitochondrial gene has been developed for the specific identification of bovine, ovine and caprine DNAs in feedstuffs. The primers designed generated specific fragments of 84, 121 and 122 pb length for bovine, ovine and caprine species, respectively. The specificity of the primers designed was tested against 30 animal species including mammals, birds and fish, as well as eight plant species. Analysis of experimental feedstuffs demonstrated that 0.1% of raw and heated bovine, ovine or caprine tissues can be easily detected using the species-specific primers developed. The performance of this method is not affected by prolonged heat treatment, and consequently it could be very useful to verify the origin of the raw materials in products submitted to denaturing technologies, for which other methods cannot be applied.

Keywords: Polymerase chain reaction; Ruminant species identification; Feedstuffs; 12S rRNA gene

1. Introduction

Bovine spongiform encephalopathy (BSE) is a progressive degenerative disease that affects the central nervous system of cattle. It was first diagnosed in Great Britain in 1986 and thought to be linked to the practice of supplementing ruminant feed with rendered protein derived from scrapie infected sheep tissues (Kusama, Nomura, & Kadowaki, 2004). Since 2002, the introduction in the European Union of active monitoring of transmissible spongiform encephalopathy (TSE) in small ruminants has detected atypical scrapie cases. The implications of atypical scrapie as distinct from scrapie are difficult to quantify in terms of its impact on animal health due to insufficient data (EFSA, 2005b). More recently, BSE has been confirmed in goats, and the Scientific Panel on Biological Hazards of the European Food Safety Authority (EFSA) indicated that important information gaps remain related to the quantification of risk to human health associated with consumption of goat meat (EFSA, 2005a).

The atypical scrapie cases, the appearance of BSE in goats and the emergence of the new variant of Creutzfeldt–Jacob disease in humans, has focused attention on the need of an adequate control of animal feedstuffs. Cross contamination by feed containing animal proteins and intended for non-ruminants is considered to be the main remaining source of BSE infection since the introduction of the ruminant feed ban in 1994. Regulation (EC) N° 999/2001 of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies prohibits the feeding of animal protein to farmed animals, with the exception of certain animal proteins (Regulation 01/999 EC; Anon, 2001). Therefore, the detection of animal tissues in feedstuffs is an issue of

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doi:10.1016/j.meatsci.2006.06.019

great significance for the implementation of measures against the spread of BSE (Lahiff et al., 2001) and to verify compliance with labelling requirements and feed traceability. The concept of traceability through the food supply chain is recognized within the European Union with the regulation (EC) N° 178/2002, in which traceability is defined as the ability to trace and follow food, feed and ingredients through all stages of production, processing and distribution (Regulation 02/178 EC; Anon, 2002a). Thus traceability requires systems to detect adulterations or fraudulent substitutions in feedstuffs (Orrú, Napolitano, Catillo, & Moioli, 2006).

The presence of meat and bone meals (MBM) in feed can be demonstrated by microscopic examination. The microscopic method, based on the detection of animal bone fragments, has been recognized as the official method in the European strategy against the BSE (Directive 98/88 EC; Anon, 1998). However, it is time consuming, requires specialized staff and only enables the detection of zoological classes, while the species origin of bone fragments remains undetermined (Dalmasso et al., 2004).

Currently, 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. Protein-based techniques include electrophoretic, chromatographic and immunological assays. Although most of these protein-based methods identified the species of origin of raw meats, they were significantly less sensitive in heat-treated material because of the alteration of the specific epitopes (Hofmann, 1996). In the case of immunoassays, only antibodies raised against heat-stable biomarkers can be used for detection of MBM in animal feed (Kim et al., 2004, 2005).

The disadvantages of protein-based methods can be solved with methods based on the presence of species-specific DNA sequences, detected by techniques such as the polymerase chain reaction (PCR). The amplification of specific DNA sequences by PCR provides a rapid, sensitive and specific method for detection of animal tissues in food and feed (Arslan, Irfan Ilhak, & Calicioglu, 2006; Meyer, Candrian, & Lüthy, 1994).

Many PCR-based methods cannot be used for detection of MBM, because the high temperatures involved in the standard rendering process, cause fragmentation of DNA, leading to difficulty in obtaining reliable results (Colgan et al., 2001; Frezza et al., 2003). Because of this, species identification in samples where DNA can be severely degraded must rely on amplification of short DNA targets (Krcmar & Rencova, 2005).

Most assays of feedstuffs test only for bovine material and will not detect tissues from other ruminant species (Bottero et al., 2003; Krcmar & Rencova, 2001; Myers et al., 2001; Tartaglia et al., 1998; Toyoda, Nakajo, Kawachi, Matsui, & Yano, 2004; Wang et al., 2000). In this article we describe the development of a species-specific PCR method based on the 12S ribosomal RNA mitochondrial gene, for the detection and identification of bovine (*Bos taurus*), ovine (*Ovis aries*) and caprine (*Capra hircus*) tissues in feedstuffs. The specificity of the primers designed was tested against 30 animal species including mammals, birds and fish, as well as eight plant species.

2. Materials and methods

2.1. Sample selection

Muscular, nervous and fatty tissues from bovine, ovine and caprine species were provided by a local slaughterhouse. Raw meat samples from horse, pork, rabbit, turkey, chicken, duck and goose were purchased from local markets. Muscle samples of cat, dog and rat tissues were obtained from The Veterinary Hospital (Facultad de Veterinaria, Universidad Complutense de Madrid, Spain). Muscle samples from red deer, fallow deer, roe deer, and chamois were obtained from several Spanish meat-cutting plants located at the Comunidad de Madrid (Parque Natural "El Pardo"), Andalucía (Parque Natural Sierra de Cazorla, Segura y Las Villas) and Castilla-La Mancha (Ciudad Real and Toledo). Fish samples like tuna, anchovy, wreck fish, sole, sea bass, hake, grouper, nile perch, monkfish, atlantic salmon, sardine, and rainbow trout were purchased form local markets.

All animal specimens were morphologically identified by trained veterinarians. They were transported to the laboratory under refrigeration, and processed immediately or stored frozen at −85 °C until used.

Heat treated samples from bovine, ovine and caprine species (muscles, nervous tissue and fatty tissue) were processed in an autoclave in compliance with European legislation (Regulation 02/1774 EC; Anon, 2002b). Three different heat treatments were applied, as follows:

Treatment 1: 120 °C for 50 min.
Treatment 2: 110 °C for 120 min.
Treatment 3:133 °C at 3 bar for 20 min.

Samples of plant species (oats, barley, maize, rye, wheat, sunflower and soybean) were purchased from local markets.

In order to evaluate the test sensitivity, nine binary mixtures of animal tissues in a plant matrix were prepared (bovine tissues in oats, ovine tissues in oats and caprine tissues in oats). Five different percentages, 0.1, 1, 5, 10 and 25% (w/w) were prepared for each species and tissue (muscle, nervous and fatty tissue) in the oats matrix. Mixtures were made in a final weight of 100 g using a blender (Sunbeam Oster, Florida, USA). Moreover, pure samples of the target species (100%) were used as positive controls.

The effect of thermal treatments on the technique's ability to identify the target species was checked through the analysis of binary mixtures prepared as described above, but using the different percentages of the heat treated animal tissues.

All the binary mixtures were stored at −20 °C until used.