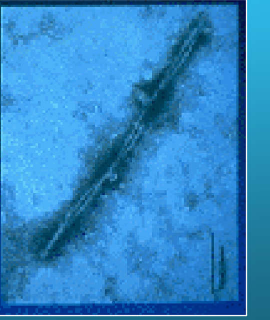
DETECTION OF BOVINE DNA IN FEED BY REAL-TIME PCR

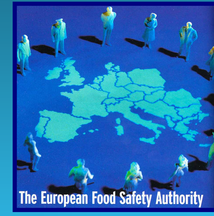


Martín, I., García, T., González, I. and Martín, R.

Departamento de Nutrición, Bromatología y Tecnología de los Alimentos.

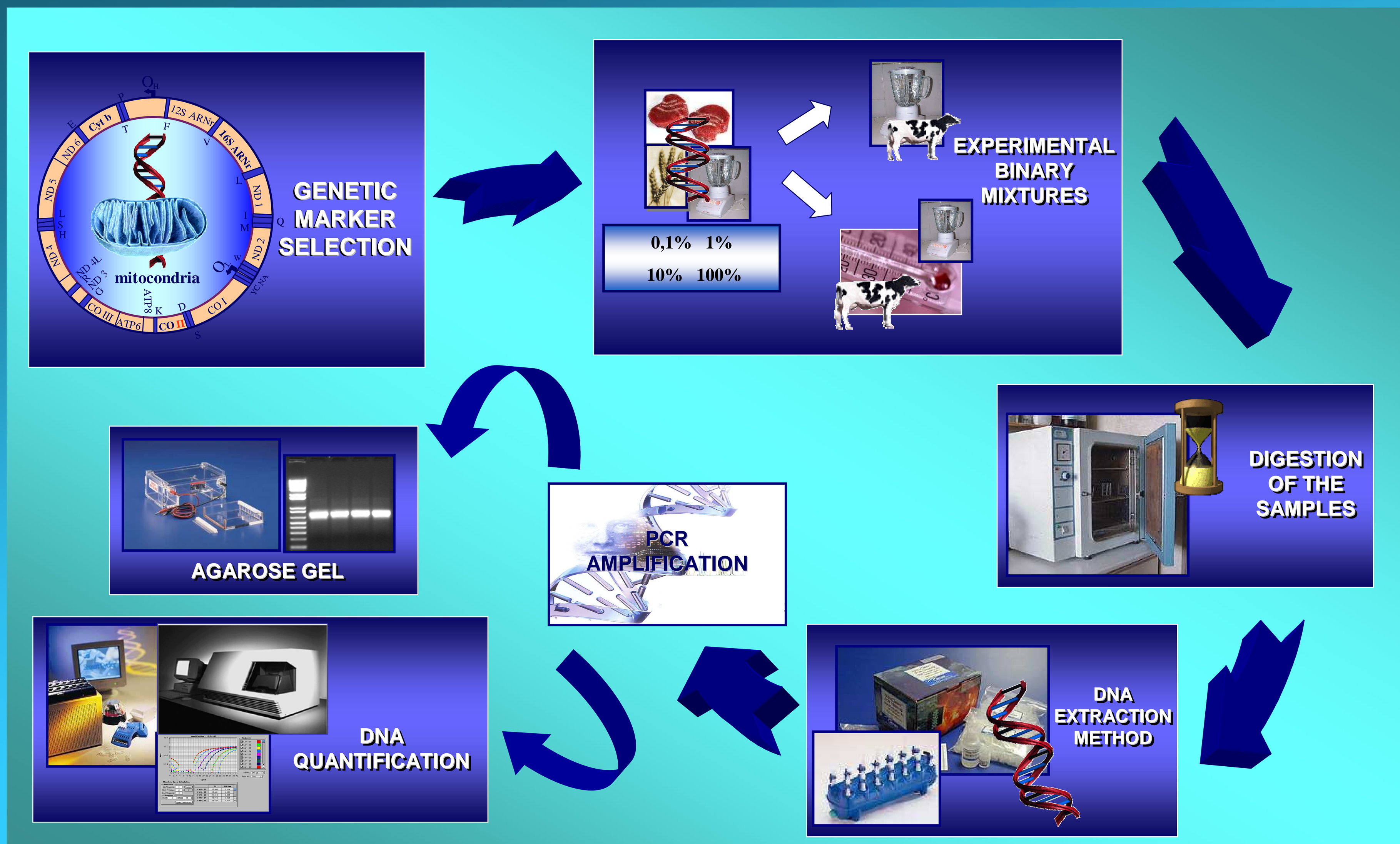
Facultad de Veterinaria. Universidad Complutense de Madrid. 28040, Madrid. España.

INTRODUCTION



The outbreak of bovine spongiform encephalopathy (BSE), and the emergence of the new variant of Creutzfeldt-Jacob disease have demonstrated the importance of implementation strict regulations in the composition of feedstuff. Currently, the official method used in the European Union for determination of prohibited materials in animal feed is microscopic examination (European Commission, 2003). This method is very effective in identifying the presence of minute amounts of meat and bone material. However, microscopy does not allow for determination of the species origin of the material, and is time-consuming and labor-intensive (Myers, 2005). Because of its heat stability, DNA is a suitable target for species identification assays. PCR analysis of species-specific mitochondrial DNA sequences is the most common method currently used to identify animal tissues in food and animal feedstuffs. The aim of this work has been to develop a real-time PCR approach for the detection of bovine DNA in feedstuffs.

MATERIALS AND METHODS



The method combines the use of bovine-specific primers, that amplify a 84 bp fragment of the mitochondrial 12S ribosomal RNA gene, and universal primers that amplify a 140 bp fragment of the nuclear 18S ribosomal RNA gene from eukaryotic DNA. The 18S rRNA primers are used as endogenous control for the total content of PCR-amplifiable DNA in the sample.

DNA was extracted using the *Wizard® DNA Clean-up system* (Promega, Madison, WI, USA), as described by Martín et al., 2007. The PCR reactions, using the Power SYBR Green PCR Master Mix (Applied Biosystems), were run with the ABI Prism 7700 sequence detection system (Applied Biosystems).

The amount of bovine DNA of the samples was normalized to an endogenous reference that is the 18S rRNA gene sequence, and expressed as $2^{-\Delta\Delta Ct}$ using the comparative *Ct* method (User Bullettin #2, ABI Prism 7700 SDS, Applied Biosystems).

The PCR efficiency and quantification limits were evaluated not only from the standard curve made with serial dilutions of bovine DNA, starting from 100 ng, but also from raw and heat treated experimental binary mixtures containing different percentages (100, 10, 1 and 0.1%) of bovine tissues (muscle or fat) in an oats matrix. The heat treated mixtures were processed in autoclave in compliance with European legislation at 133º for 20 min at 300 kPa (European Commission, 2002).

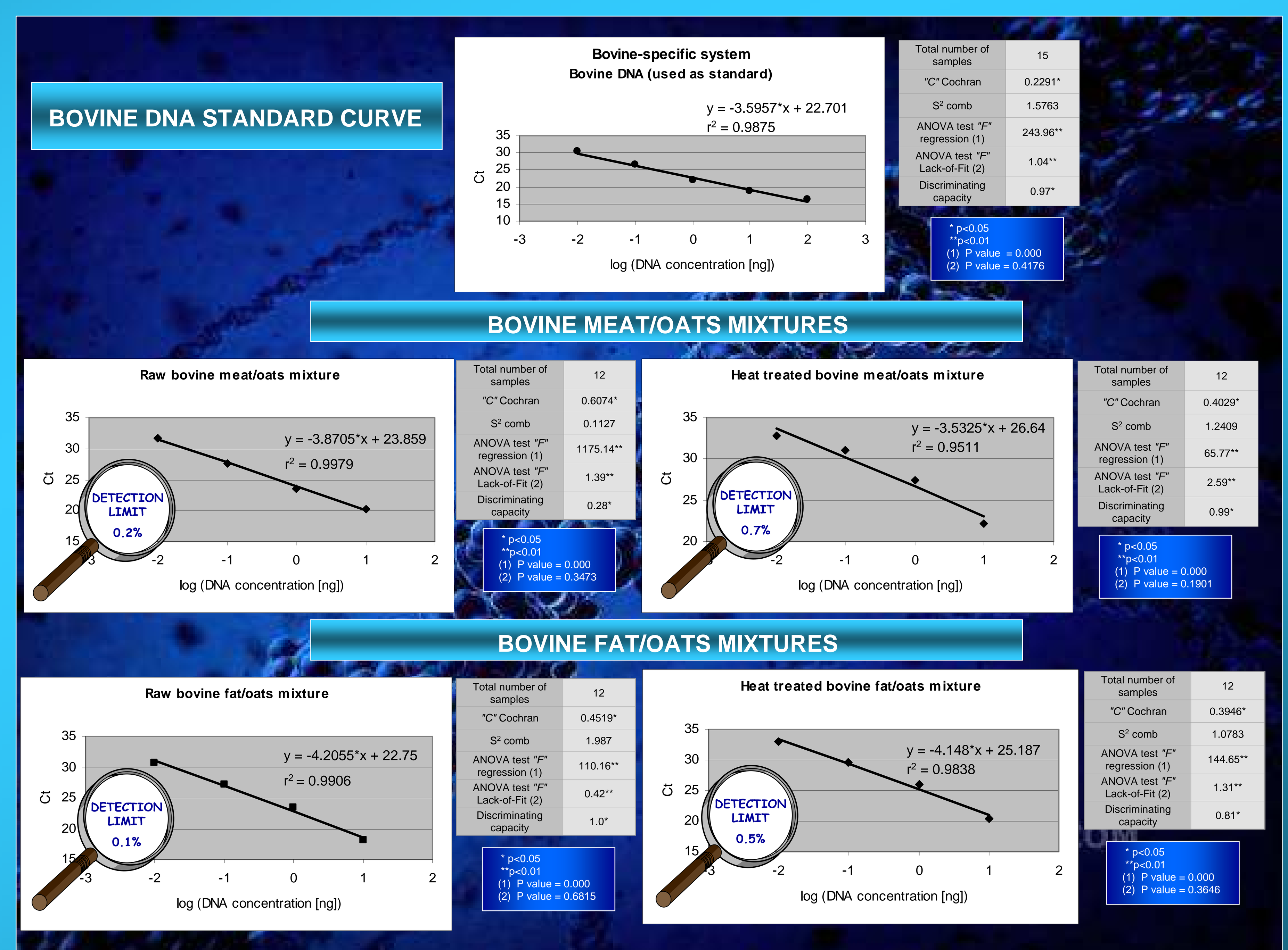
RESULTS

The specificity of the primers was tested against 18 animal species including mammals, birds and fish, as well as 6 plant species. The bovine-specific system amplified a 84 bp fragment from bovine DNA (Ct value of 17.23 ± 0.09), with a melting temperature (Tm) between 78.5 and 79ºC, while no homologous product was amplified from any other animal or plant DNA tested (Table 1). Ct values above that corresponding to the mean Ct value of all the non-target species and negative controls minus twice their standard deviation (Ct > 30.52) were considered negative for the presence of bovine DNA. The eukaryotic system amplified a 140 bp fragment from all the eukaryotic species analysed. All tested species showed Ct values between 18.42 and 14.25 (Table 1), and a Tm between 83 and 84ºC.

TABLE 1. Specificity of the quantitative PCR systems (C_t alues obtained from 10 ng DNA).

Name	Scientific name	*SPECIFIC SYSTEM*	*EUKARYOTES SYSTEM*
Cattle	*Bos taurus*	17.23±0.09	16.63±0.11
Sheep	*Ovis aries*	33.24±1.56	16.83±0.82
Goat	*Capra hircus*	35.28±1.25	17.50±0.47
Pig	*Sus scrofa domestica*	33.04±1.99	15.56±1.12
Chicken	*Gallus gallus*	34.66±2.56	16.76±0.59
Turkey	*Meleagris gallipavo*	34.63±0.38	15.23±0.56
Duck	*Anas platyrhynchos x Cairina muschata*	35.89±0.47	15.18±0.70
Goose	*Anser anser*	34.80±0.83	15.87±0.75
Horse	*Equus caballus*	35.01±3.88	17.32±3.85
Rabbit	*Oryctolagus cuniculus*	35.62±1.40	18.31±3.50
Cat	*Felis catus*	36.03±1.51	18.42±0.60
Dog	*Canis familiaris*	34.90±0.65	15.58±0.77
Rat	*Rattus norvegicus*	35.49±0.87	16.87±0.62
Anchovy	*Engraulis encrasicolus*	35.23±1.23	16.37±0.81
Atlantic salmon	*Salmo salar*	37.25±1.61	15.01±0.05
Four spotted meagrim	*Lepidorhombus boscii*	38.49±1.30	14.94±0.11
Grouper	*Epinephelus marginatus*	33.68±0.97	14.25±0.09
Sardine	*Sardina pilchardus*	35.10±1.58	16.11±0.78
Tuna	*Thunnus spp*	33.47±0.60	15.93±0.26
Barley	*Hordeum vulgare*	31.96±0.43	16.03±0.55
Maize	*Zea mays*	32.10±0.16	14.60±0.57
Oats	*Avena sativa*	36.71±2.61	16.75±0.57
Soybean	*Glycine max*	35.75±2.09	15.57±0.44
Rye	*Secale cereale*	33.20±0.54	15.58±0.61
Wheat	*Triticum aestiuum*	34.16±0.81	14.61±0.12

To carry out the validation of the technique, linearity test, regression line parameters and sensitivity parameters of the bovine-specific SYBR Green PCR system were evaluated in the standard curve and in the binary mixtures.



Analysis of experimental bovine tissues/oats mixtures demonstrated the suitability of the assay for the detection of bovine DNA in mixtures in the range between 0.1-0.7 to 10% depending on the tissue and treatment of the sample, as long as the calibrator sample used is made with the same tissue and treatment as the samples.

CONCLUSION

The reported real-time PCR assay may represent an accurate and efficient procedure for detecting minute amounts of bovine DNA in raw and heat treated food and feedstuffs as long as the reference sample used has received the same treatment than the samples analyzed.

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