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SYBR-Green real-time PCR approach for the detection and quantification of pig DNA in feedstuffs

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ABSTRACT

A real-time polymerase chain reaction assay using primers targeting the porcine-specific mitochondrial 12S rRNA gene and universal eukaryotic primers amplifying a conserved fragment of the nuclear 18S rRNA gene has been developed for the detection and quantification of porcine DNA in food and feedstuffs. The 18S rRNA primers were used as endogenous control for the total content of PCR-amplifiable DNA in the sample. The assay was tested on DNA extracted from raw and heat-treated binary mixtures of porcine tissues in a plant matrix, and on DNA extracted from reference feedstuff samples. Analysis of experimental mixtures demonstrated the suitability of the assay for the detection and quantification of porcine DNA in mixtures containing as little as 0.1%.

1. Introduction

The ban on using processed animal proteins (PAPs) as feed additive for all farm animals is an important measure to prevent the spread of Transmissible Spongiform Encephalopaties (TSE). Currently, this ban is regulated by Council Decision 2000/766/EC (European Commission, 2000). Moreover, the animal byproduct (ABP) regulation 1774/2002/EC (European Commission, 2002) imposed a ban on feeding animals with proteins from the same species.

Enforcing both legislations requires the availability of reliable analytical methods that show specific performance characteristics depending which aspect of the PAP ban are addressed. For instance, the requirements from the ABP regulation (European Commission, 2002), show that there is a need for methods that allow detecting the presence of species-specific PAPs (e.g. PAPs from pig).

In 1998 the microscopic method was validated by an intercomparison study thereby becoming the only official method (European Commission, 1998, 2003) suitable for the determination of PAPs in feed. This official microscopic method has its limits for the discrimination at the level of vertebrate classes. No discriminating characters are present at lower taxonomic levels (e.g. ruminant versus non-ruminant). In this context, the development of alternative techniques for the identification of the species present in meat and bone meals (MBMs) is necessary.

Recently, a range of analytical approaches have been developed broadly based on detecting either protein or DNA molecules. Among the first, immunoassays are the most widely used techniques. The main advantages of the applied immunoassays are the good sensitivity along with a capability of a high sample throughput. A major problem seemed to be its limitation when trying to identify the species in highly processed foods, since proteins are denaturated during heat-treatments, high pressures and other processing technologies (Chen, Hsieh, & Bridgman, 2004). For this reason immunological analyses have been replaced by DNA-based methods. DNA has the advantage of being a relatively stable molecule, is more able to withstand heat and pressure processing and its sequence is conserved within all tissues of an individual (Lanzilao, Burgalassi, Fancelli, Settimelli, & Fani, 2005).

The limitations of standard PCR assays include the lack of quantitation of end-point analysis, and the dependence on a low throughput technique such as agarose gel electrophoresis for analysis of the products. Conversely, by means of real-time PCR approaches, even minute traces of different animal species can be discriminated and measured in foods and feeds of complex composition.

The development of a real-time PCR assay for the detection and quantification of porcine DNA content in porcine tissues/oats binary mixtures and feedstuffs is reported. The approach is based on

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Table 1
Real-time PCR analysis of reference feedstuff samples using porcine-specific primers. (C_p values produced using 10 ng DNA).

Sample	Heat-treatment (°C)	Chicken MBM% (w/w)	Actual porcine content		C_p values [b]	Porcine content detected [c] (w/w)	
			Description	% (w/w)		(%)	Coefficient of variation (CV)
A	159	0	Porcine bones	100[a]	36.28 ± 0.89	*U*[d]	–
B	159	0	Porcine soft tissues	100	27.80 ± 0.16	3.68	10%
C	133	0	Porcine soft tissues	100	22.70 ± 0.23	103.58	15%
D	133	99	Porcine carcase and muscle	1	28.93 ± 0.08	1.76	5%
E	133	99.9	Porcine carcase and muscle	0.1	33.57 ± 0.11	0.08	7%
F	133	99	Porcine MBM	1	30 ± 0.21	0.87	14%
G	133	99.8	Porcine MBM	0.2	32.10 ± 0.24	0.22	16%
H	133	99.9	Porcine MBM	0.1	33.31 ± 0.22	0.10	15%

[a] Content as percentage of porcine tissues is given for the total weight.
[b] C_p values are expressed as mean ± standard deviation.
[c] Detection (+) was defined by a C_p of lower than 35.53.
[d] *U* indicates uncertain result. Some replicates above and other below the cut-off value.

selective PCR amplification of DNA fragments on the 12S ribosomal RNA mitochondrial gene.

2. Materials and methods

2.1. Sample selection

Samples of pork, beef, goat, sheep, horse, rabbit, turkey, chicken, duck and goose were provided by a local slaughterhouse. Muscle samples from cat, dog, and rat were obtained from The Veterinary Hospital (Facultad de Veterinaria, Universidad Complutense de Madrid, Spain). Fish and plant species samples were purchased from local markets.

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

In order to evaluate the test sensitivity, two independent binary mixtures of muscle or fat porcine tissues in a plant matrix were prepared. For each mixture, four different percentages of 0.1, 1, 5 and 10% (w/w) of muscle or fat porcine tissues in oats were prepared in a final weight of 100 g. Two hundred milliliter of sterile phosphate-buffered saline (PBS; 136 mM NaCl, 1.4 mM KH_2PO_4, 8.09 mM $Na_2HPO_4{\cdot}12H_2O$, and 2.6 mM KCl, pH 7.2) were added to the binary mixtures, and they were homogenized using a blender (Sunbeam Oster, Florida, USA). A pure sample of the target species (100% pork) was used as positive control.

The effect of thermal treatments on the technique's ability to identify the target species was checked through the analysis of binary mixtures prepared with heat-treated tissues. To prepare the heat-treated mixtures, 100 g of porcine muscle or fat were finely chopped and processed in an autoclave in compliance with European legislation (European Commission, 2002) at 133 °C for 20 min at 300 kPa. Temperature was checked by introducing a temperature data logger, mod EDI-85A/125A (Ebro Electronic GMBH & Co, Ingolstadt, Germany) in the autoclave, together with the muscle or fat. Once the tissues were autoclaved and left to cool at room temperature, they were used to prepare the porcine tissues/oats mixtures containing 0.1 to 10% porcine component. Raw and heat-treated binary mixtures were processed directly or stored at −20 °C until used.

Feedstuff samples containing a known percentage of porcine tissues (Table 1) were provided by CCL Nutricontrol (Feed to Food Quality Services, Veghel, The Netherlands).

2.2. DNA extraction

Genomic DNA was obtained from 200 mg of animal, plant, feedstuff or binary mixture materials using the *Wizard® DNA Clean-up system kit* (Promega Corp, Madison, WI, USA), as described by Martín et al. (2007). Three separate extractions of each percentage (0.1, 1, 5, and 10%) were prepared for all the binary mixtures, and were analyzed. DNA concentration was estimated by UV absorption spectrophotometry at 260 nm.

2.3. Primers design

The porcine-specific primers used in real-time PCR were designed based on 12S rRNA gene sequences from various animal and plant species available in the Genbank database, using Primer Express 2.0 software (Perkin-Elmer/Applied Biosystems Division, Foster City, CA, USA). A second set of primers (*18SFWEU-18SRVEU*) was used as endogenous control for the detection of eukariotic DNA. The sequences and description of the primers used in this work are summarized in Table 2.

The primer *set 12SCerdDIR* and *12SCerdINV* was designed for the specific amplification of a 75 bp fragment of the 12S rRNA gene. The target site for the endogenous PCR system consisted of a 99 bp fragment of the 18S rRNA. Results obtained from the endogenous PCR system were used to normalize those obtained from the specific systems as described below.

2.4. PCR conditions

The optimum PCR concentrations of primers yielding the highest end-point fluorescence and the lowest C_p were experimentally determined for each set of primers (porcine-specific and endogenous control system). Optimum concentration per reaction was

Table 2
DNA sequences of the primers used in this study.

Primers	Length (bp)	Sequence (5′ to 3′)	Used as
12SCerdDIR	18	CCTCCTCAAGCATGTAGT	Porcine-specific forward primer
12SCerdINV	23	GTTACGACTTGTCTCTTCGTGCA	Porcine-specific reverse primer
18SFWEU	21	AGGATCCATTGGAGGGCAAGT	Eukaryotes forward primer
18SRVEU	25	TCCAACTACGAGCTTTTTAACTGCA	Eukaryotes reverse primer