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Analytical Methods

Avian-specific real-time PCR assay for authenticity control in farm animal feeds and pet foods

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ABSTRACT

A highly sensitive TaqMan real-time PCR assay targeting the mitochondrial 12S rRNA gene was developed for detection of an avian-specific DNA fragment (68 bp) in farm animal and pet feeds. The specificity of the assay was verified against a wide representation of animal and plant species. Applicability assessment of the avian real-time PCR was conducted through representative analysis of two types of compound feeds: industrial farm animal feeds ($n = 60$) subjected to extreme temperatures, and commercial dog and cat feeds ($n = 210$). Results obtained demonstrated the suitability of the real-time PCR assay to detect the presence of low percentages of highly processed avian material in the feed samples analysed. Although quantification results were well reproducible under the experimental conditions tested, an accurate estimation of the target content in feeds is impossible in practice. Nevertheless, the method may be useful as an alternative tool for traceability purposes within the framework of feed control.

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1. Introduction

The compound feed industry consists of two distinct sectors: farm animal feeds and pet foods. Within these sectors, authenticity and related correct product labelling are essential issues to prevent frauds and to avoid potential safety risks caused by the introduction of any food ingredient that might be harmful to human and/or animal health (Casazza, Gavazzi, Mastromauro, Gianì, & Breviario, 2011; Pascoal et al., 2011). A relevant example of this is the public health crisis caused by the bovine spongiform encephalopathy (BSE) disease, which was linked to the consumption of animal feed contaminated by infected ruminant proteins. To restore consumer's confidence, a total ban on the feeding of processed animal proteins (PAPs) to all farmed animals (with limited exceptions), was enacted since January 2001 (Regulation 999/2001/EC). Although the ban is still in place, a partial amendment is being under debate by European authorities during the last few years towards the re-entry of certain non-ruminant proteins (i.e. pig and poultry proteins) into feed for mono-gastric animals, assuming that the transmission risk of BSE from non-ruminants to non-ruminants is "very unlikely" (EFSA Panel on Biological Hazards (BIOHAZ), 2011). A related concern of European authorities regarding traceability of feed materials is that affecting the use of animal by-products for manufacturing pet foods. Nowadays, dog and/or cat owners often do not have the time, money and resources to devote to preparing fresh wholesome meals for their pets, thus relying upon the use of the different kinds of processed food available in the market (Kang & Kondo, 2002). To comply with legislation, pet food labels must indicate either all the ingredients present in the product or the categories to which the ingredients belong, and should be transparent, consistent, coherent and understandable (Regulation 767/2009/EC).

In spite of the existence of mandatory traceability requirements for farm animal and pet feeds, labels do not provide sufficient guarantee about the real species and nutrient composition of a product. These limitations highlight the need for reliable analytical techniques to enforce the legislative frame and allow the authentication of animal feedstuffs (Chiappini et al., 2005; Goffaux, China, Dams, Clinquart, & Daube, 2005). Techniques based on the analysis of the molecular components of cells, either proteins or DNA, are widely applied for species identification in food for human and animal consumption. However, their performances are dependent on processing conditions used at manufacture and the heterogeneous composition of the materials under study (Frezza et al., 2003; Momcilovic & Rasooly, 2000). Protein-based methods, such as immunoassays, are limited since the targets of detection are denatured proteins. Consequently, species identification will not be optimal in matrices subjected to food processing procedures involving severe heat treatments (Bottero & Dalmasso, 2011). Conversely, DNA is an extremely stable molecule that can be recovered from biological material that has been under stress conditions, like processed feedstuffs. Moreover, DNA is found in the majority of tissues of an organism and can provide more information than proteins due to the degeneracy of the genetic code and the presence of

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large non-coding regions (Lockley & Bardsley, 2000; Pereira, Carneiro, & Amorim, 2008).

In the last decade, a variety of reliable and specific DNA-based methods have been developed for food and feed species authentication (Bottero & Dalmasso, 2011; Fumière et al., 2009). Amplification of specific DNA sequences by means of the polymerase chain reaction (PCR) is presently the most widely used genetic approach for species assignment in feeds (Fumière et al., 2009). Among PCR-dependant approaches, real-time PCR is based on the detection of fluorescence produced after the accumulation of PCR products with each cycle of amplification. The ability of measuring the reaction kinetics in the early phases of PCR provides a distinct advantage over other PCR methods, providing the analyses with automation, large-scale processing and quantitative potential (Dalmasso, Civera, la Neve, & Bottero, 2011; Prado et al., 2007). Real-time PCR data can be obtained using a number of different chemistries, mainly TaqMan and SYBR Green technology. In a TaqMan assay, a probe is used that specifically binds to the target amplicon. Thus, an increased specificity is achieved relative to a normal two-primer PCR or non-specific binding dyes such as SYBR Green (Ballin, Finn, Vogensen, & Karlsson, 2009; Lockley & Bardsley, 2000).

The objective of the present work was to develop a TaqMan real-time PCR method for selectively detect avian DNA in feeds for farm animals and pets, targeting short mitochondrial 12S rRNA gene sequences specific to the avian-class. The applicability of the assay was evaluated through the analysis of a wide representation of industrial farm animal feeds subjected to extreme temperatures (up to 145 °C), plus an extensive batch of commercially processed pet food products.

2. Materials and methods

2.1. Sample selection

Authentic samples from a wide range of animal and plant species from different origins were included in the assays for specificity control purposes. Samples were obtained from several local abattoirs and retail markets (Madrid, Spain). Target avian samples consisted of muscle tissues from various species belonging to the anseriformes, galliformes and struthiformes orders (chicken, turkey, quail, pheasant, partridge, guinea fowl, duck, goose and ostrich, Table 1).

Farm animal feed test materials used in the present study were provided by the Co-operative Central Laboratory "CCL-Nutricontrol" (Veghel, The Netherlands) and consisted of 60 industrial PAPs containing concentrations from 0% to 100% of different avian tissues: bones, carcase, muscle, feather and intestines. Feeds were produced under strict controlled and recorded processing conditions. Avian material was treated at 125, 133, 137, 141 and 145 °C during 20 min, at hyperbaric pressure (Table 2).

In addition, a total of 210 commercial cat and dog food products of distinct brands and characteristics were purchased from several retail markets (Table 3).

2.2. DNA extraction

Genomic DNA was obtained from 200 mg of each sample using the Wizard DNA Clean-up System kit (Promega, Madison) as described by Pegels et al. (2011). The DNA was eluted in 100 μL of sterile deionised water and its concentration and quality was measured by at 260 and 280 nm wavelengths using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Montchanin, USA). Unless otherwise stated, three DNA replicates were extracted from each sample. A negative control sample was included in every DNA extraction.

2.3. Primers and probes design

Mitochondrial 12S rRNA gene sequences from various animal species available in the NCBI (National Center for Biotechnology Information) database were aligned with the EMMA programme included in the EMBOSS software package version 2.0. After sequence comparison and analysis, an avian-specific primer pair was designed for the selective amplification of a 66–69 bp DNA fragment common to the aligned avian species (Fig. 1), which were selected on the basis of their possible use in animal feed preparation and on DNA sequence availability. The primers were *A12SDIR*: 5′-CAGTGAGCTCAATAGCMCC-3′ and *A12SINV*: 5′-CCCATTTCTYCCACCY-3′. Degenerate oligonucleotides were introduced along primer sequences in order to potentially detect all the avian species considered without affecting assay specificity. A TaqMan probe, *A12SP*: 5′-6FAM-GATATGGA + ACT + G + G + ACAGAA -BBQ-3′ was designed to anneal within the gene fragment generated by amplification of the corresponding avian target. Besides, a conserved 18S rRNA primers/probe system previously reported by Pegels et al. (2011) was used as positive amplification control of the assay. Symbols (+) along the oligonucleotide sequences denote LNAs (locked nucleic acid bases) which were needed for optimal probe hybridisation. Primer Express 2.0 software (Perkin-Elmer/Applied Biosystems Division, Foster City, CA) was used to help on the primer design. TaqMan probes were designed and synthesised by TibMolBiol (Berlin, Germany).

2.4. Real-time PCR amplification

Real-time PCR was run under generic cycling conditions. The optimum PCR concentrations of primers yielding the highest endpoint fluorescence and the lowest crossing point (C_p) value were 900 nM for each forward and reverse primer. The PCR reactions were carried out using the LightCycler TaqMan Master (Roche Diagnostics GmbH, Mannheim, Germany), 2 pmol of each TaqMan probe (TibMolBiol), and 2 μL of extracted DNA. Amplification reactions were performed in a total reaction volume of 10 μL in a glass capillary tube and were run on the LightCycler 2.0 Instrument (Roche Applied Science, Penzberg, Germany) with the following programme: 10 min at 95 °C, an amplification programme of 45 cycles at 95 °C for 10 s, 53 °C for 30 s, and 72 °C for 1 s. Samples were finally cooled to 40 °C for 30 s. This programme was used to amplify the avian-specific system together with the positive amplification control. Unless otherwise indicated, all real-time PCR reactions were carried out in triplicate for each DNA extract.

The C_p value, which refers to the cycle number where the sample's fluorescence significantly increases above the background level, was calculated automatically by the LightCycler software as the first maximum of the second derivative of the curve.

2.5. Construction of the standard curve

To assess the efficiency, linear range and analytical sensitivity of the avian real-time PCR system, two sets of the reference industrial animal feeds (Table 2: bones, R1–R4; carcase, R6–R10) rendered under homogeneous conditions (133 °C) and containing increasing amounts of the target material were used to construct separate standard curves. The amount of target DNA in an unknown sample can be then measured by extrapolation of the C_p value obtained in the unknown sample in the corresponding standard curve of C_p values generated from known DNA percentages of the target species. In addition, the correlation between the variables, C_p and concentration ([ ]) is semilogarithmic:

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