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Real-time PCR for quantitative meat species testing

Jason Sawyer [1], Clare Wood [2], Della Shanahan [3], Sally Gout, David McDowell *

Department of Life Sciences Research, LGC, Queens Road, Teddington, Middlesex TW11 OLY, UK

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Abstract

A method for quantitative meat speciation is described which combines the use of real-time PCR with species specific and 'universal' primers to measure individual species content and total meat content respectively. A comparison of the cycle number at which universal and species specific PCR products are first detected, in combination with the use of reference standards of known species content, is used as the basis for determining the percentage of a given species in a mixed sample. Importantly, the use of universal primers allows differences in DNA quality between samples and reference standards to be taken into account, while the use of real-time PCR allows measurement at an early stage in the PCR process which is inherently more accurate than the end point analysis associated with gel-based systems. This paper describes the quantification of beef in mixed samples to illustrate the principle of this approach.

Keywords: Authenticity; Quantification; Real-time PCR

1. Introduction

The need to support food-labelling legislation has provided a driving force in the development of methods for the analysis of food ingredients. Meat species identification is a common requirement for regulatory enforcement laboratories and, as a result, there has been considerable research effort directed towards the development of suitable tests. To fully support enforcement activity, analytical methods should ideally be capable of quantitative measurement particularly in those cases where a certain level of adventitious contamination is permitted by legislation.

DNA methods such as hybridisation (Hunt, Parkes, & Lumley, 1997; Janssen, Hagele, Buntjer, & Lenstra, 1998), PCR-RFLP analysis (Meyer, Höfelein, Lüthy, & Candrian, 1995; Wolf, Rentsch, & Hübner, 1999), PCR assays (Matsunaga et al., 1999; Meyer, Candrian, & Luthy, 1994), SSCP analysis (Rehbein, Kress, & Schmidt, 1997) and sequencing (Bartlett & Davidson, 1992) have complemented more established protein-based techniques (Barai, Nayak, Singhal, & Kulkarni, 1992) in an attempt to provide reliable, robust and simple to use speciation strategies. All these techniques have particular advantages and disadvantages and may be selected according to the particular requirements and characteristics of the sample under analysis. Generally, these offer, at best, semi-quantitative measurement. However, hybridisation can be experimentally laborious and requires specialised training and expertise.

As already noted, PCR has been utilised for animal species identification by several authors. The amplification potential of PCR means that the technique can be exceptionally sensitive giving scope for the analysis of samples which, because of their low levels of target DNA, could not be tested using other methods. This sensitivity is, however, potentially problematic since a low level of adventitious contamination is often permitted by food labelling legislation. A common argument against the use of PCR based techniques has been that they are too sensitive and that minute traces of material would produce a positive result. There is

* Corresponding author. Tel.: +44-020-8943-7346; fax: +44-020-8943-2767.
E-mail addresses: clare.wood@hybaid.com (C. Wood), djs@lgc.co.uk (D. Shanahan), sg@lgc.co.uk (S. Gout), dgm@lgc.co.uk (D. McDowell).
[1] Present address: Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, UK.
[2] Present address: Hybaid Ltd., Action Court, Ashford Road, Middlesex TW15 1XB, UK. Tel.: +44-1784-425010; fax: +44-1784-248-085.
[3] Tel.: +44-020-8943-7444; fax: +44-020-8943-2767.

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therefore a requirement to develop quantitative PCR tests that are sufficiently accurate to distinguish samples containing adventitious levels of contamination from those that are deliberately mis-described or adulterated.

This paper describes the development of a method for quantitative meat identification based on combining the use of real-time PCR (Heid, Stevens, Livak, & Williams, 1996; Higuchi, Dollinger, Walsh, & Griffiths, 1992; Higuchi, Fockler, Dollinger, & Watson, 1993; Wittwer, Hermann, Moss, & Rasmussen, 1997), a technique which offers great scope for the development of quantitative PCR assays, with the use of universal and species specific PCR primer pairs. A comparison of the cycle number at which universal and species specific PCR products are first detected, in combination with the use of reference standards of known species content, is used as the basis of determining the percentage of a particular species in a mixed sample.

The use of universal primers also allows the degradation state and quality of the DNA recovered from a sample to be taken into account by allowing comparison of its amplification response with that of reference standards. This is important given the variable condition of DNA recovered from food samples which can result in unpredictable amplification. The use of real-time PCR also offers advantages by allowing measurement at an early stage in the PCR process which is inherently more accurate than the end point analysis typically associated with gel based measurement.

To illustrate the principles of this approach, the development of a system for the quantitative determination of beef in mixed samples is described.

2. Materials and methods

2.1. Preparation of meat admixtures

Tissue from prime muscle cuts of beef and lamb, obtained from local suppliers, was mixed in appropriate ratios to a total of 5 g. DNA was then extracted as described below.

2.2. DNA extraction

DNA was extracted from meat samples as previously described (Hunt et al., 1997).

2.3. Design of universal primers P and Q

Cow (Anderson et al., 1982) and chicken (Desjardins & Morais, 1990) mtDNA 16S rRNA genes were aligned using the Align program (Lasergene software; DNASTAR) and primer sites chosen against conserved sites at positions 2770–2789 (UNIV P primer) and positions 2855–2874 (UNIV Q primer) of the published cow mtDNA sequence (Anderson et al., 1982) to give a PCR product size of 104 bp. Primer sequences are shown in Table 1. Cow and chicken sequences were selected as being distantly related with regions of conservation likely to be maintained between more closely related species.

2.4. Design of cow specific PCR primers

Mitochondrial DNA control region sequences from cow (Anderson et al., 1982), sheep (Zardoya et al., 1995), pig (Ghivizzani, MacKay, Madsen, Laipis, & Hauswirth, 1993) and goat (Sawyer, 1996) were aligned using the Align program (Lasergene software; DNASTAR). Regions of species divergence were identified and primer sites selected to exploit these differences and enable specific amplification from a particular species (Sawyer, 1996). The primer sites selected were at positions 101–120 (COW 1 primer) and positions 318–338 (COW 2 primer) in the published cow mtDNA sequence (Anderson et al., 1982) to give a PCR product size of 237 bp with the addition of a single-stranded tail from the Scorpion primer.

A Scorpion primer (Whitcombe, Theaker, Guy, Brown, & Little, 1999) was used to allow the accumulation of the cow specific PCR amplification product to be monitored. The cow specific Scorpion primer was designed and synthesised by Oswel Ltd. and incorporated the cow specific primer sequence which had been identified as described above. Accumulation of universal primer amplification product was measured with SYBR Green I (Higuchi et al., 1992). Primer sequences are shown in Table 1.

2.5. PCR amplification

DNA (10–50 ng) was amplified using the LightCycler (Roche Diagnostics) (Wittwer et al., 1997) and Light-

Table 1
PCR primers used in this study

Name	Primer sequence
UNIV P	5′-GGTTTACGACCTCGATGTTG-3′
UNIV Q	5′-CCGGTCTGAACTCAGATCAC-3′
COW 1	5′-ATTGACTTTGTTTGGAGTGCT-3′
COW 2	5′-FAM CCGCGGCATGCAGTTAAGTCCAGCGCCGCGG-MR-HG
(Scorpion)	GGCCCTGACCCGGAGCATCT-3′

FAM is 6-carboxy-fluorescein, HG is hexaethylene glycol and MR is methyl red monomer.

Cycler—DNA Master SYBR Green I and Hybridization Probes kits (Roche Diagnostics) for universal and species specific reactions respectively following the manufacturer's instructions. Amplification was carried out in glass capillaries in a final volume of 20 μl containing 4 mM $MgCl_2$. The samples were subjected to the following thermal cycling protocol: denaturation (95 °C for 30 s); amplification (25–30 cycles of 95 °C for 0 s, 55 °C for 5 s, 72 °C for 10 s at a ramp rate of 20 °C/s). Samples were then cooled to 40 °C. Universal primer reactions were amplified with primers UNIV P and UNIV Q; beef specific reactions were amplified with primers COW 1 and COW 2 (Scorpion) detailed in Table 1.

2.6. Quantitative analysis using the LightCycler

Following PCR, the data was analysed using the LightCycler quantitation software. The 'fit points' method was used, typically with proportional baseline adjustment, following the manufacturer's instructions, to display the real-time PCR traces and calculate the cross point values. In order to maximise comparability between controls and samples, a normalisation process was undertaken. This involved a preliminary analysis using the same nominal amounts of input DNA which were then adjusted in a repeat analysis in order to achieve a similar C_T value for this target.

3. Results and discussion

In an effort to produce a robust method for quantitative meat speciation, the use of species specific primers, universal primers and real-time PCR was combined into a single method.

To illustrate the experimental principles of this system, a beef in lamb admixture series (containing 0.1–100% beef) was amplified in the LightCycler with universal and beef specific primers. The universal primers were designed to amplify DNA from all the common meat species and produced a similar response regardless of the species content for the samples under investigation (Fig. 1). In contrast, amplification with the beef specific primers resulted in an earlier cross point value or first detection point (the cycle number at which an amplification signal is first recorded) with increasing beef content.

A major advantage of this method is that factors affecting such as inhibition and degradation can be taken into account when carrying out quantitative measurements. This is achieved through the use of appropriate dilutions of the reference standards and unknown samples such that they can be matched or normalised to produce an equivalent amplification response from the universal primers.

In order to determine the beef content in unknown samples, the unknown samples and reference standards (which contain a known amount of beef) are amplified with the beef specific primer set and the amplification signals compared. Without the use of universal primers it would be difficult to be certain whether variations in species specific primer response were due to differences in species content or other factors such as DNA degradation, inhibition or differences in the amount of DNA added to the PCR. By allowing both comparative measurement (species specific versus universal signal)

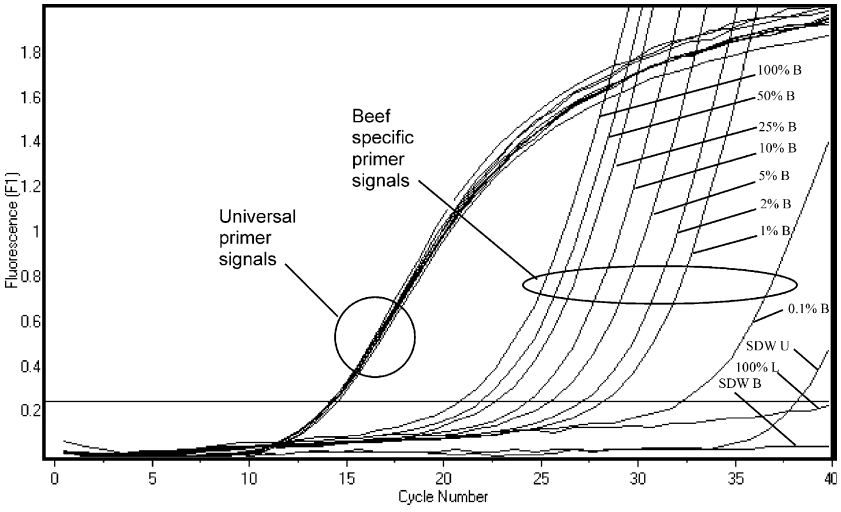

Fig. 1. Measurement of beef in a beef:lamb DNA admixtures series by real-time PCR analysis. A DNA admixture series containing beef (B) and lamb (L) (0.1%, 1%, 2%, 5%, 10%, 25%, 50% and 100% beef in lamb) was amplified using universal primer and beef specific primer sets. Real-time accumulation of PCR amplification products was measured using the LightCycler machine employing SYBR Green I and Scorpion probe detection methods for the universal and beef specific primers respectively. SDW B and U indicates water added to the PCR reactions in place of DNA for the beef specific and universal primer mixes respectively. The late signal from the universal reactions following this number of cycles is normal with SYBR Green I detection due to the formation of primer dimers.

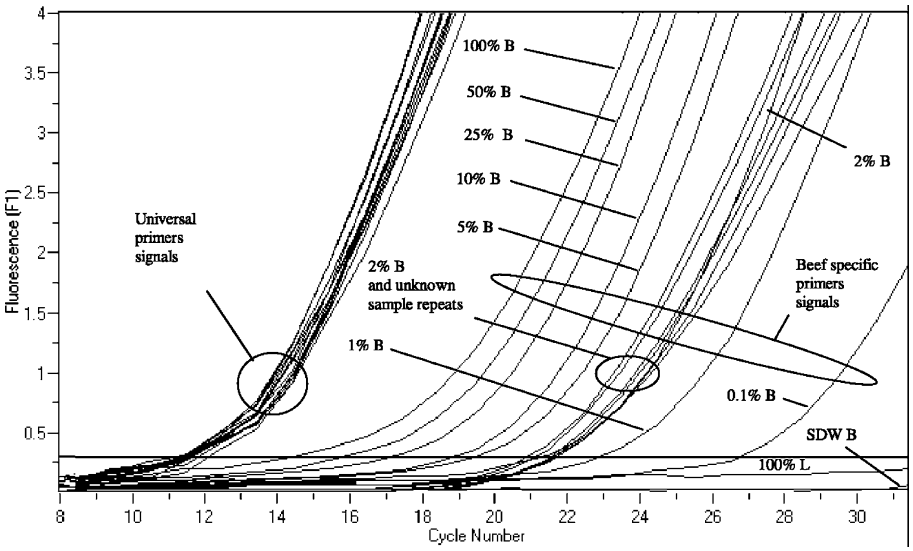

Fig. 2. Measurement of beef in a sample (known to contain beef and lamb) by real-time PCR analysis. The unknown sample was amplified alongside a DNA admixture series containing beef (B) and lamb (L) (0.1%, 1%, 2%, 5%, 10%, 25%, 50% and 100% beef in lamb) using universal and beef specific primers. Real-time accumulation of PCR amplification products was measured using the LightCycler machine employing SYBR Green I and Scorpion probe methods for the universal and beef specific primers respectively. 'Blank' indicates water added to the PCR reactions in place of DNA.

and normalisation of amplification response between unknowns and reference standards, it is hoped that this approach will reduce the inaccuracies caused by the use of control and unknowns that, by definition, are not identical.

In theory, the ratio of species specific and universal primer response should be constant for any particular sample, irrespective of concentration and the degradation/inhibition state of the DNA, because the amplification of both sets of primers should be affected to the same extent. This would be convenient in terms of not having to match samples with unknown samples. However, in preliminary work (data not shown) we found that this was not necessarily the case and, for example, the change in amplification response with increasing DNA concentration was not constant for universal and species specific primer sets.

Analysis of a minced lamb sample suspected of containing beef (Fig. 2) demonstrates the practical application of the method. Matching of the amplification response with universal primers and subsequent amplification and comparison of the mince DNA against a beef in lamb admixture series with the beef specific primers (Fig. 2) indicated that the sample contained approximately 2% beef.

The work described in this paper has shown that the combined use of real-time PCR together with universal and species specific primers shows promise for the development of a system for quantitative meat speciation. Although improvements in the assay system can be envisaged (for example, amplification and detection of both targets in a duplex reaction and the development of a universal Scorpion probe to allow a single system for fluorescent detection) this work has illustrated both the principle and utility of this approach.

The accuracy of the method could be affected by factors such as the degree of degradation and the presence of different tissue or species types potentially containing different numbers of mitochondria or yielding different amounts DNA. Further critical testing of the method on suitable control and blind samples as well as comparison with other DNA speciation methods will be essential to assess the accuracy of the technique and determine if it will prove to be a method suitable for the routine analysis of quantitative meat species analysis.

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Clare Wood was awarded a BSc. in Molecular and Cellular Biology from the University of Bath (1996) and then a MSc. in Forensic Science from Kings College London (1998). This was followed by work in the Life Sciences research team developing DNA based approaches for meat speciation testing. She presently works as a technical specialist at Hybaid Ltd.

Della Shanahan gained a BSc. in Biochemistry at John Moores University, Liverpool in 1990. Since then she has worked in the Life Sciences Research Team at LGC where she has been involved in a large variety of projects primarily concerned with the application of molecular biology techniques to food analysis.

Sally Gout obtained a BSc. in Molecular Biology from the University of Manchester before completing a MSc. in Forensic Science at Kings College, London. Since joining the Life Science research group at LGC in 1997 she has worked primarily on projects developing tissue specific PCR assays and quantitative meat speciation assays.

Jason Sawyer was awarded a BSc. in Biological Science from Stirling University in 1991 before completing a PhD in Genetics at Leicester University. He worked at the Brewing Research Foundation (yeast and bacterial genetics) for two years before moving to LGC in 1997. At LGC he worked on a variety of projects including development of molecular methods for animal speciation and GMO detection. He moved to the Veterinary Laboratories Agency in September 2000.

David McDowell gained a BSc. in microbiology from Imperial College, London and went on to complete a PhD at Warwick University on the plasmid biology of *Bacillus thuringiensis*. After microbiological post-doctoral work, he joined the LGC Life Sciences Research group. At LGC he has worked on and managed a diverse range of projects including quantitative PCR method development, validation of molecular biology techniques and the development of tissue specific PCR assays.