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# Identification of the species of origin of raw and cooked meat products using oligonucleotide probes

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A simple assay suitable for the routine determination of species composition in admixtures of meat is described. A nonradioactive slot blot hybridisation assay using species-specific oligonucleotide probes has been developed and applied to the species identification of rabbit, sheep, pork, beef and goat meats. Clear species discrimination was demonstrated even between the closely related ruminants goat and sheep. The probes were shown to identify species present in both raw and commercially cooked and canned products (e.g. petfood). The potential for semi-quantitation of species in admixture was demonstrated to a detection limit of less than 2.5% adulteration. This DNA assay targets intracellular DNA and can therefore overcome the potential problem of blood and plasma drip contamination which has led to uncertainty when using soluble immunoassays directed towards soluble plasma protein. © 1997 LGC (Teddington) Ltd. Published by Elsevier Science Ltd

## INTRODUCTION

Fraudulent substitutions of alternative meat species in meat products have led to the need for reliable and specific methods of meat species determination. A variety of analytical approaches have been described in the literature, predominantly based on protein analysis or immunological assay (Patterson & Jones, 1990). The main disadvantage of the detection of proteins as the characterising component of the natural ingredient or adulterant is that their expression is tissue dependent and they may be denatured on processing and heating, leading to subsequent loss of analytical specificity. Consequently, such techniques have limited application to highly processed foods. DNA is a relatively stable molecule, and the specific recognition of characteristic sequences of DNA within the sample by a suitably labelled complementary DNA sequence, the probe, can form the basis of a uniquely specific assay. The potential of such a DNA-based analytical approach employing a simple slot/dot blot assay format has been investigated (Baur *et al.*, 1987; Wintero *et al.*, 1990;Chikuni *et al.*, 1990; Ebbehof & Thomsen, 1991*a*). These authors have reported the use of total genomic species DNA as the probe: pork and chicken were clearly identified, however, specific ruminant species identification was not possible due to high sequence homology resulting in cross-hybridisation between closely related species. This approach has therefore proved insufficiently specific for a generally applicable analytical method. Attempts to overcome this problem by competition with unlabelled DNA (Ebbehof & Thomsen, 1991*b*) were of limited success, with discrimination between the more closely related ruminants, sheep and goats, remaining unsatisfactory.

Another DNA-based approach to species identification employing the polymerase chain reaction (PCR), (Saiki *et al.*, 1988) has been described for raw and cooked meat species identification using growth hormone gene specific primers (Meyer *et al.*, 1993). A modification of the technique using sheep satellite I DNA primers for PCR amplification followed by restriction digestion of the product has enabled discrimination between sheep and goat meat samples (Chikuni *et al.*, 1994). Meyer *et al.*, 1995, have also employed post-PCR restriction digest analysis for meat species determination. However, these PCR-based techniques are relatively expensive, technically demanding and prone to contamination. Although a powerful

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technique when sample quantity is limiting and extreme sensitivity is required, the sophistication of PCR is not generally necessary for routine species identification in foods. Furthermore, when determining meat species levels in admixtures, the sample is not usually limited and the amount taken for DNA extraction needs to be representative of the mix in the sample, therefore it is preferable to start with a larger amount of tissue (typically 1-5 g). Methods for quantitative PCR are still in need of further development for this type of application (Ferre, 1992; Reischl & Kochanowski, 1995; Raeymaekers, 1995).

There is still clearly a need for a routinely applicable, reliable and specific method of analysis for meat species identification in raw and highly processed products for use by food analysts who do not have access to PCR equipment. The overall aim of this work programme was to develop a simple nonradioactive slot blot-based analysis which can be successfully applied to the specific and semi-quantitative identification of a variety of meat species in admixture, and to evaluate its performance against a range of immunological procedures. A disadvantage of the majority of commercial immunological methods is that they detect soluble plasma proteins, and it has been argued that this is not 'meat' and may arise from adventitious contamination with blood from other species. The described DNA assay, however, targets intracellular DNA and when combined with a sample wash procedure allows detection of DNA from intact muscle cells thus eliminating the possibility of cross - contamination.

Species-specific oligonucleotide probes have been developed which hybridise to relatively short sequences of DNA, therefore a considerable amount of DNA degradation can be accommodated without influencing assay specificity. The successful application of this method for the specific qualitative identification of species in admixture in highly processed and canned meat products is also demonstrated.

## MATERIALS AND METHODS

### Samples

Raw meat samples and canned and processed meat products for analysis were obtained from local retailers and processed immediately. Authentic species blood samples were obtained through the Royal Veterinary College, London.

### Materials

All chemicals used were of Molecular Biology or AR grade. Ribonuclease A Type 1-A was obtained from Sigma Chemical Company and Proteinase K from BRL, Life Technologies Inc., Biodyne B membrane from Pall Process Filtration Ltd, Hyperfilm-ECL from Amersham International, *Hind III* and *Xba I* restriction enzymes from Pharmacia Biotech.

Nonradioactive probe labelling reagents: digoxigenin DNA labelling and detection kit, digoxigenin ddUTP, digoxigenin oligonucleotide 3′ end labelling kit and the chemiluminescent substrate CSPD® were obtained from Boehringer Mannheim Ltd.

### Assay protocol

A schematic representation of the key steps in the slot blot assay is presented in Fig. 1.

### Sample DNA preparation

Total genomic DNA was extracted, as described by Sambrook *et al.*, 1989, from unprocessed tissue samples from pig, sheep, cattle, goat and rabbit, and also from a variety of cooked, canned and processed meat products. 20 ml of extraction buffer (10 mM Tris, 100 mM EDTA and 0.5% w/v sodium dodecylsulphate (SDS)) was added to approximately 5 *g* of sample in a 50 ml tube and vortexed briefly followed by 2 min on a roller mixer. The sample was centrifuged at 1203 *g* for 1 min and the supernatant removed by decantation. This step was included to lyse and remove any cellular or plasma material which may have adventitiously contaminated the sample from another source. 20 ml of extraction buffer was added to the pellet and homogenised to a smooth consistency using a Camlab Homogeniser Omni 5000. The sample was treated with ribonuclease A added to a final concentration of 20 $\mu$g/ml for 1 h at 37°C followed by an incubation with proteinase K at a

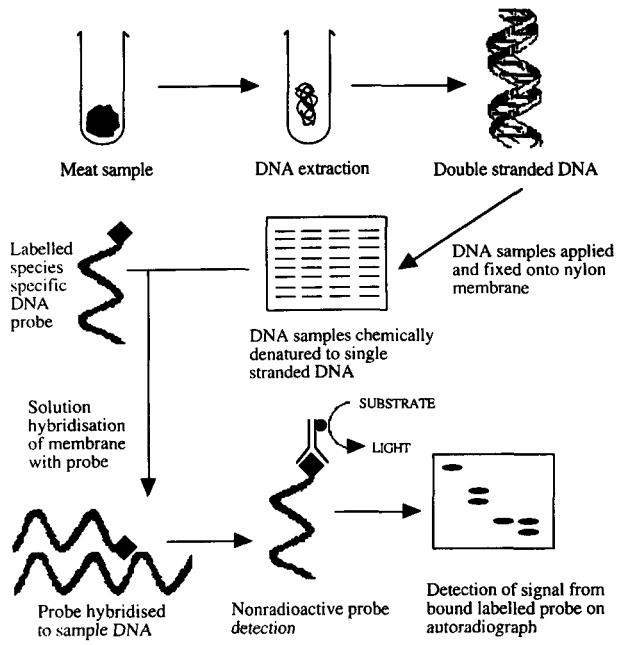


**Fig. 1.** Schematic representation of key steps in the nonradioactive slot blot assay for meat species identification.

final concentration of 100 $\mu$g/ml for 2 h at 52°C. The sample digest was then purified until a minimal protein interface was obtained by a series of equal volume extractions of 0.1 M Tris pH8 equilibrated phenol, phenol/chloroform/isoamylalcohol (24:24:1) and chloroform/isoamylalcohol (24:1). 0.2 volumes of 10M ammonium acetate and 2 volumes of absolute ethanol were added to precipitate the DNA from the final aqueous phase. The DNA pellet was recovered by centrifugation at 3900 *g* for 45 min, the pellet was washed with 2 ml of 70% ethanol and finally resuspended in 0.5–1.0 ml of $TE_8$ (10 mM Tris, 1 mM EDTA pH 8).

**Assessment of quality and concentration of DNA**

The concentration of the extracted DNA was determined by reading the absorbance at 260 nm ($A_{260}$) using UV spectroscopy (Sambrook *et al.*, 1989). The molecular size and quality of the DNA was determined by agarose gel electrophoresis. The DNA extraction method employed here ensures a high yield of optimum quality DNA for hybridisation analysis.

**Preparation of slot blots**

The sample DNAs to be analysed were loaded onto a Biodyne B membrane (500 ng-lug DNA per slot) using a slot blot manifold (Biorad). The membranes were then treated with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min followed by neutralising solution (1.5 M NaCl, 0.5 M tris, 0.001 M EDTA, pH7.2) for 1 min. The membranes were air dried.

**DNA probes**

The porcine satellite DNA specific probe was a 600 bp insert excised from the plasmid pSRIC (Davies *et al.*, 1988) using *Hind III* and *Xba I*. Oligonucleotide probes specific for rabbit, goat, lamb and beef meats were derived from the species satellite DNA sequences (Demers *et al.*, 1986; Buckland, 1985; Novak, 1984; Pech *et al.*, 1979). Nucleotide sequences were aligned and fragments (approximately 50 bases in length) of least homology identified using MegAlign on Lasergene DNA*. The specificity of these defined nucleotide sequences was then confirmed by further computer nucleic acid database analysis using the EMBL and GENBANK databases on DNA*. Defined oligonucleotide sequences were synthesised commercially at King's College School of Medicine and Dentistry, London.

The porcine probe was nonradioactively labelled by random priming with digoxigenin-11-dUTP (Feinberg & Vogelstein, 1983). Oligonucleotide probes were also nonradioactively labelled according to the suppliers' instructions, using the digoxigenin oligonucleotide 3′ end labelling kit.

**Hybridisation and washing conditions**

Each slot blot was prehybridised for at least 1 h at 68°C in a 5 ml solution of 5xSSC, 1% (w/v) blocking agent (Boehringer Mannheim), 0. 1% (w/v) N-lauroylsarkosine and 0.2% (w/v) SDS. The specific digoxigenin labelled probe was added to each prehybridisation solution to a final concentration of 10-50 pmol/ml and incubated for 1 h at 54°C. After hybridisation nonspecifically bound probe was removed in a series of stringency washes by incubating the membranes at 65°C twice in 6xSSC, 0.1% SDS for 5 min followed by once in 2xSSC, 0.1% SDS for 5 min.

Specifically bound probe was visualised by chemiluminescent detection using an antidigoxigenin-alkaline phosphatase conjugate and CSPD® as described by the suppliers. The threshold level of signal detection is dependant on the properties of the X-ray film and was enhanced by preflashing the film to an OD of 0.15 using a flash gun with an 'Orange' Kodak Wratten No. 21 acetate filter. This increases its sensitivity and linearises its response at low light signal levels (Laskey & Mills, 1975). The chemiluminescent signal from hybridised probe was visualised by exposure to preflashed X-ray film, typically for 15–60 min. The intensity of the developed autoradiographic, signals which are proportional to the percentage of the target meat DNA present, were measured using a BioImage gel and autoradiograph documentation and densitometric analysis system, BioImage, UK. Calibration graphs were produced from the data using CA-Cricket Graph III software. Quantity estimations by simple visual examination of the hybridisation signal from the unknown sample(s), in comparison with signals from reference admixture standards on the same blot were also possible.

**Preparation of reference admixture blots for quantitative analysis**

The DNA was extracted separately from the raw meat of lamb and beef species and then mixed to prepare a range of percentage DNA admixtures (0, 2.5, 5.0, 7.5, 10, 15% (mass/mass) lamb in beef). Three reference admixture series were prepared, each one made using different samples of lamb and beef DNA. A batch of four identical slot blots were prepared, each containing the three different admixture series (1 $\mu$g amounts of each percentage DNA admixture was loaded onto the membrane). The slot blots were hybridised individually with a different aliquot from the same batch of digoxigenin labelled ovine specific probe. Specifically bound probe was visualised by chemiluminescent detection on preflashed film after an exposure of 15 min. Hybridisation signals were measured using a BioImage gel and autoradiograph documentation and analysis system and the image intensities recorded for each percentage of the series were used to generate a calibration graph using curve fit on the graph package software.

## RESULTS AND DISCUSSION

### Probe specificity

The application of the developed slot blot hybridisation assay employing several of the derived species specific oligonucleotide probes for the analysis of DNA extracted from raw meat samples of several different species is illustrated in Fig. 2. The complete specificity of each of the oligonucleotide probes is clearly demonstrated, with no cross-species hybridisation observed with the DNA of other meat species. In particular, there was no cross-reactivity between the closely related ruminants goat and sheep, confirming the increased specificity of these probes over the total genomic DNA approach reported by other workers. Figure 2 also illustrates the detection of 5% lamb in beef and 5% pork in beef.

The potential sensitivity of the oligonucleotide probes was effectively enhanced by the fact that they were derived from the specific species satellite DNA sequences. These satellite sequences consist of repeated DNA units which are present in high copy number in the genome (eg. 27% of the total bovine genome consists of satellite DNA, Pech *et al.*, 1979). The presence of these high copy number units therefore increases the amount of target sequence available for hybridisation to the probe.

### Assay format

The results from the hybridisation of these DNA admixtures demonstrates the potential with this simple assay format for the determination of several species present in admixture. Replicate application of the sample DNA under analysis to separate columns of the slot blot, followed by hybridisation of each strip with a particular species probe, allows the determination and relative quantitation of different species present in an admixture in a particular product.

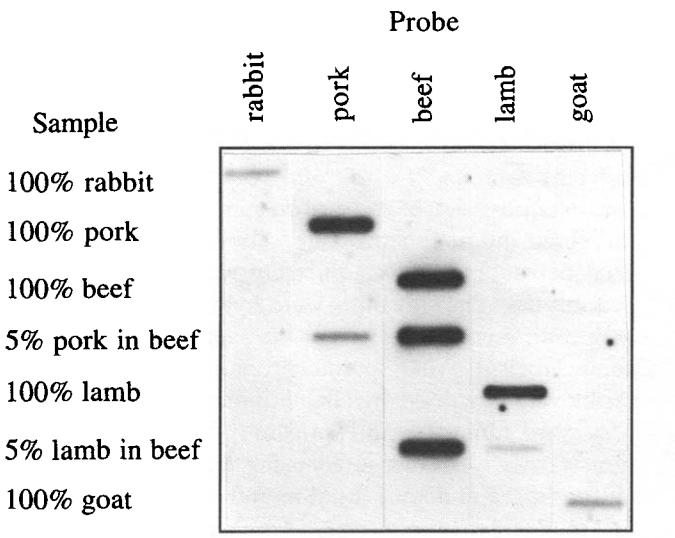


**Fig. 2.** A nonradioactive slot blot hybridisation showing specificity of species specific oligonucleotide probes.

A further advantage of the application of oligonucleotide probes as reported here is that the kinetics of the oligonucleotide hybridisation reaction enables a rapid hybridisation step, typically one h or less, (as demonstrated in Fig. 2), as compared with the overnight hybridisation generally applied when more complex total genomic DNA is used as a probe.

The results shown in Fig. 2 were obtained using digoxigenin labelled probes, with specific probe binding detected by the use of the chemiluminlescent substrate CSPD®. This method of nonradioactive labelling and detection has several advantages over the $^{32}$P-radiolabelled DNA probes reported by other workers. The digoxigenin labelled probes give comparable sensitivity of detection to radioactively labelled probes but exposure times are shorter, i.e. 15–60 min as compared with overnight or longer exposures using radioactivity. The digoxigenin labelled probes are stable on storage for at least six months and the hybridisation buffer can be

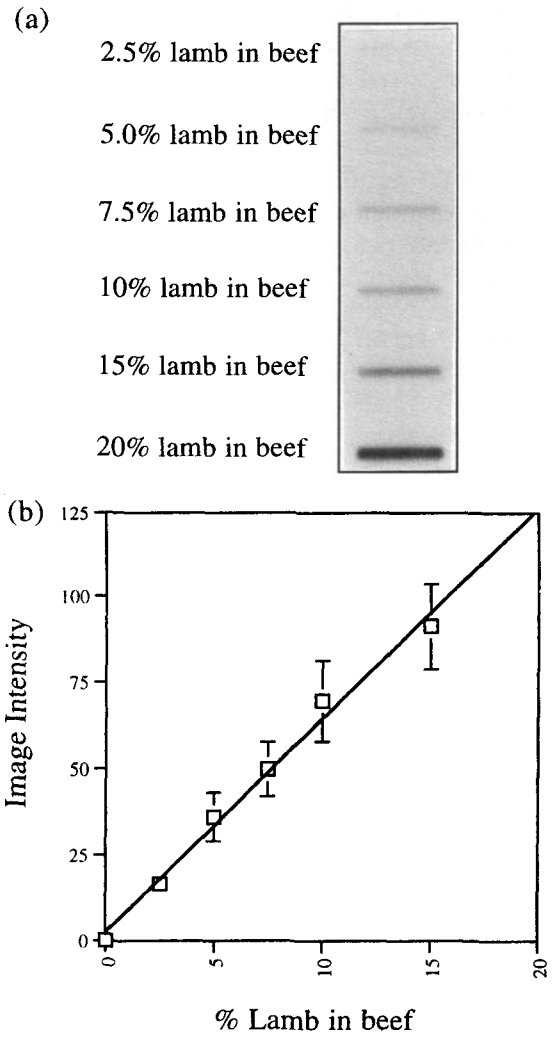


**Fig. 3.** (a) Nonradioactive quantitation of a series of increasing lamb in beef admixtures hybridised with the ovine specific probe. (b) Calibration graph of varying percentage lamb in beef produced from analysis of signal intensities of autoradiographs such as the one shown in Fig. 3(a).

stored and reused, whereas $^{32}P$ radiolabelled probes have a half-life of 14 days and are unstable on storage and cannot readily be reused. A nonradioactive assay is safer and more convenient for routine use than a radioactive assay which requires a licence, monitoring of use and disposal, and special equipment.

**Quantitation in admixture**

The potential for quantitation of species in an admixture was investigated. Figure 3a shows the nonradioactive signals obtained from one series of lamb in beef DNA admixtures. It can be seen that a positive hybridisation signal is obtained at the minimum admixture level employed, 2.5% lamb in beef, thus demonstrating a sensitivity of signal detection of less than 2.5%.

The assay reproducibility was assessed by comparison of results from three different fresh meat DNA admixture series from four separate analyses using the same slot blot hybridisation and detection conditions. Figure 3b shows a graph of the mean and standard deviation (S.D.) values of the three fresh meat DNA admixture series from the analysis of one of these slot blots. Comparing the mean values of the test 6% DNA admixture across these four slot blots gives a reproducibility within 2% (absolute). The results demonstrate that the slot blot hybridisation assay can produce a calibration graph to give semi-quantitative data.

The efficiency and yield of DNA extraction from meat samples may vary depending on the cell density, and hence DNA content, for equivalent weights of different cuts of meat, with varying muscle and fat content; this could influence quantitation results achieved and should form the basis of a further investigation.

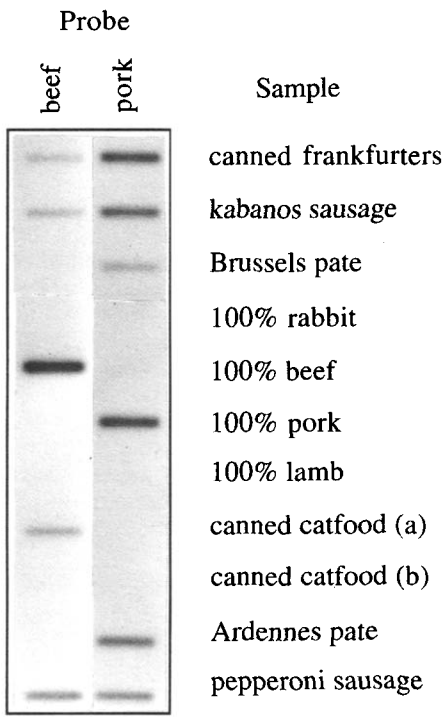


**Fig. 4.** Species identification in processed meats using bovine and porcine oligonucleotide probes. Catfood (a):- beef, rabbit and other. Catfood (b):- lamb and other.

**Qualitative analysis of cooked/processed meats in admixture**

The application of the assay for the identification of species of origin in processed meats is illustrated in Fig. 4. Duplicate slot blots were loaded with 100% control DNA and sample DNA from canned frankfurters and catfood, two pates, and pepperoni and other processed sausages. These membranes were hybridised separately with a) bovine and b) porcine digoxigenin labelled probes. These results demonstrate that the short species specific oligonucleotide probes we have described can identify the different species present in a variety of processed and canned meat products, including highly processed petfoods, even though the DNA is substantially degraded.

In cooked meat the DNA may have been exposed to high temperatures for prolonged periods which denatures the DNA. It has been reported that measured DNA concentrations from spectrophotometric readings may depend on the integrity of the DNA (Labarca & Paigen, 1980). To produce a calibration graph for the semi-quantitation of cooked meat the DNA in the admixture series should be treated so that the quality of the extracted DNA is of equivalent quality/degradation to that observed in the processed samples under analysis.

## CONCLUSIONS

Previous DNA-based approaches to species identification have included slot blot hybridisation assays employing total genomic DNA probes with slow reaction kinetics and the inability to discriminate between closely related species. Faster and more specific PCR-based approaches have also been reported, but the extreme sensitivities obtainable are not generally necessary or desirable for routine food analysis. The research community has indicated that there are many technical problems associated with PCR which are not always appreciated, e.g. contamination, variance between machines, appropriate controls, matrix effects and interpretation of data. Specialist training is required to use the technique correctly for analytical purposes and dedicated facilities are essential to minimise contamination problems. Furthermore, PCR based methods for quantitation of the extent of sample adulteration are complex and require further development for this type of application.

A nonradioactive assay for the identification of meat species has been described here which retains the simplicity of the slot blot hybridisation assay format but, by employing species-specific oligonucleotide probes complementary to repetitive satellite DNA sequences, which is capable of increased specificity and sensitivity.

The potential for semi-quantitation of species in admixture has been confirmed to a detection limit of less than 2.5% adulteration, and the application of the assay to the analysis of a wide range of commercially processed, heated and canned products has also been demonstrated. Differences in DNA extractability were observed in different tissues such as offals and lean meat from the same species (results not shown), a factor which would have to be taken into account if attempting to quantify unknown tissue mixes in admixture.

The described DNA assay has been used in parallel with a range of commercial and in-house developed immunological assay procedures for the examination of meats and meat products for a number of years in the Laboratory of the Government Chemist. This type of approach has also been applied in our laboratory for the analysis of various fish, poultry, vegetable and cereal crop based food products for which we have derived suitable oligonucleotide probes.

Further simplification of this assay is under investigation. A reverse hybridisation assay is being developed with immobilisation of the species specific oligonucleotides, which should allow the determination of several species in a particular sample in a single hybridisation event.

## ACKNOWLEDGEMENTS

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