SPECIES IDENTIFICATION IN HEATED MEAT USING DNA OLIGONUCLEOTIDE PROBES.

BUNTJER J.B.*, LENSTRA J.A.** and HAAGSMA N.*

* Department of the Science of Food of Animal Origin, Section Chemistry, University of Utrecht, The Netherlands. ** Institute of Infectious Diseases and Immunology, Recombinant DNA Facility, University of Utrecht, The Netherlands.

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SUMMARY.

The use of specific DNA oligonucleotide probes for the identification of animal species in autoclaved meat is described.

The probes are derived from satellite DNA (stDNA) sequences of turkey, chicken, horse and pig respectively. StDNA consists of species specific, non-coding, tandem-repeated units with a high copy number. The probes are coupled directly to alkaline phosphatase as reporter group. After a rapid DNA extraction from autoclaved meat samples, extract is spotted on nylon membranes and the blots are hybridized to the probes. The amount of bound probe is detected with a chemiluminescent substrate.

The test was able to detect 1 % of meat of other species mixed in meat samples and to discriminate between closely related species. Autoclaving the meat samples for 90 min or more, decreased the hybridization signal.

Practical and methodological aspects of the hybridization procedure are discussed.

Introduction.

Species identification in meat products is of importance for economical, religious, or public-health reasons.

Most tests for the identification of animal species in meat products are based on protein analysis. Several techniques to detect species specific protein differences in raw or processed meat have been reviewed by Hitchcock *et al.* (1985) and Kaiser *et al.* (1985). The majority of techniques are based on electrophoretical principles or immunological detection. Recently DNA hybridization tests for species identification were developed.

The main problems of tests based on electrophoresis of proteins are the standardization and interpretation of the electrophoresis patterns (Hitchcock *et al.*, 1985), intraspecific polymorphisms or lack of interspecific variation (Lundstrom, 1983), and changes in the patterns if meat has been heated above 100°C (Kaiser *et al.*, 1985).

Immunological techniques (ELISA, immunodiffusion) for species identifications are sensitive and fast, but are less suitable for denatured antigens in heated meat samples. With antisera against thermostable muscle proteins 1 % admixture of other species in meat samples could be detected (Kang'ethe *et al.*, 1987, Sherikar et al., 1993). However crossreactions between closely related species as goat, sheep and cattle were found.

Winterø *et al.* (1990) and Ebbehøj and Thomsen (1991a, 1991b) described the use of hybridization of genomic DNA to DNA isolated from heated meat samples. DNA hybridization was at least as sensitive in detection of species as protein-based techniques. Less than 0.1 % admixture of meat of another species could be detected. Again crossreactions between closely related species (sheep and goat) were observed. Antonisse and Janssen (1989) and Chikuni *et al.* (1990) described the use of non-radioactive genomic DNA probes.

In the present study we describe a DNA speciation assay that incorporates the following innovations:

1. A rapid extraction of DNA from the meat samples without the use of toxic reagents.
2. The use of selective oligonucleotide probes that are based on the abundant and species-specific satellite DNA (stDNA) sequences. StDNA consists of non-coding tandem repeated sequences, situated in the centromeric heterochromatin of chromosomes (Pardue, 1975). It accounts for 5-20 % of the genomes of higher eukaryotes. The repeat units are more conserved among each other than between species (Dover, 1986). Previous studies indicated a rapid evolution of stDNA in vertebrates (Wijers *et al.* (1993), Lenstra *et al.*

(manuscript in preparation), which allows their use as species-specific probes. In addition these probes may be very sensitive because of the high copy number of the stDNA repeat units.
3. A direct coupling of the probes to alkaline phosphatase. This allows a rapid detection of the hybridized probe with the chemiluminescent substrate AMPPD™ (3-(2'-spiroadamantane)-4-methoxy-4-(3"phosphoryloxy)-phenyl-1,2-dioxetane). Probes labelled with digoxigenin or biotin must be coupled to antibody-alkaline phosphatase conjugates before detection.

The advantages and limitations of the described procedure are discussed.

Materials and Methods.

Probes.

Oligonucleotides were synthesized on a Gene Assembler 4 DNA synthesizer. Sequences of the probes were derived from satellite sequences (table I). Oligonucleotides were conjugated to alkaline phosphatase using the Lightsmith™ II kit (Promega, Madison, USA) as prescribed by the manufacturer.

Meat samples.

Raw meat samples of ca 10 g were autoclaved at 120°C for 15 min and frozen until use. Meat mixtures were made from ca 0.15 g autoclaved meat. DNA was extracted by heating 0.15 g of the meat samples at 100°C in 2 ml 0.5 M NaOH/g meat for 7 min. After two centrifugations at 13000 g for 2 min, the supernatant was used for the preparation of blots.

Blots.

An aliquot of DNA extract (5 µl) was spotted on 0.25 cm^2 Hybond N^+ nylon membrane (Amersham International, Amersham, UK) presoaked in 0.5 M NaOH. After spotting, the membrane was neutralized in 0.5 M Tris/HCl; 1.5 M NaCl, pH 8.0 and the DNA was crosslinked by UV exposure (0.72 J/cm^2, 254 nm).

Hybridization.

Blots were prehybridized with Quantum Yield™ blocking solution (Promega, Madison, USA) at 50°C for 30 min, hybridized in Quantum Yield™ high stringency hybridization buffer at 50°C for 30 min and washed two times in 2xSSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) at 50°C for 10 min. After equilibration in 100 mM Tris/HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5, the remaining alkaline phosphatase was detected with AMPPD™ (3-(2'-spiroadamantane)-4-methoxy-4-(3"phosphoryloxy)-phenyl-1,2-dioxetan, Promega, Madison, USA). Fuji RX X-ray film was exposed to the blots at room temperature for 60 min.

Results.

Specificity and sensitivity.

The hybridization signal of the meat extract of horse and chicken meat with the HMSR and the GMRS probes, respectively, was compared with the signal of purified DNA (Fig.1). A minimum of 1 ng purified DNA could be detected. The signal of the meat extracts corresponded with ca 5 ng/µl DNA. This indicates that the DNA yield of the rapid extraction is sufficient for detection by hybridization. In the standard procedure 5 µl of extract was used for the detection of admixed meat.

To test the specificity of the probes, the DNA in extracts of horse, pig, chicken and turkey meat was crosslinked to membrane pieces and hybridized to the HMSR, SSAS, GMRS and MMRS probes. As shown in Fig.2 the probes are specific for the species the sequence was derived from. Under conditions of lower stringency, slight crosshybridizations between the MMRS probe and chicken DNA were observed (results not shown).

Next meat samples of two related animals, chicken and turkey, were admixed in percentages ranging from 90 to 1 % to the other species. Hybridization of extracts of these mixtures to the GMRS and the MMRS probes, respectively, allowed the specific detection of admixture of 1 % (Fig.3).

Effects of prolonged heating and acid treatment.

As DNA is known to be degraded by long exposure to heat or acid (Ebbehøj and Thomsen, 1991a, Meyer *et al.*, 1993), the influence of these factors on hybridization signals was investigated. Raw turkey meat was autoclaved at 120°C for periods ranging from 45-120 min. After autoclaving for 90 min or longer a reduced but still clear hybridization signal was obtained. Other turkey samples that had been autoclaved for 15 min were incubated in 5 % acetic acid for 48 hours under occasional shaking. The signal of the acid treated meat was only slightly less than that of nontreated meat (Fig.4).

As an illustration, we tested the procedure on commercial meat products. Samples of lean smoked bacon, luncheon meat (declared as pork and chicken) and smoked ring sausage (declared as pork and poultry) were extracted, and hybridized to the SSAS, GMRS and HMSR probes. In the first two samples the declared meat species were identified while pork and chicken were found in the sausage. Hybridizations to the HMSR probe were all negative (Fig.5).

Discussion.

Our results indicate that the described techniques allow a sensitive and selective test for the identification of species in heated meat. The test is applicable for closely related species (chicken and turkey) that cannot be differentiated by genomic probes. Compared to published methods, the procedure is safe (no toxic reagents are used) and fast (results are obtained in 3-4 hours). Under optimal conditions, the detection limit is approximately 1 % admixture of meat of other species. Allowing for some background signals of the membrane (Fig. 1 and 5), we expect the test to have an assay limit of 5 % admixture in practice. Reduction of the background is under investigation. Presently the test is a qualitative assay only. The amount of alkaline phosphatase immobilized on the blots appeared not sufficient for a quantifiable signal with the ELISA substrate para-nitrophenylphosphate (results not shown). A restriction of any DNA test is the degradation of DNA by heating or curing of meat. In earlier described hybridization tests signals were reduced by heating (Ebbehøj and Thomsen, 1991a). In a PCR test no signal was found after 45 min 120°C or acid treatment (Meyer *et al.*, 1993). We found that hybridization signals of the short oligonucleotide probes (20-22 bp) are reduced after 90 min heating, but still allow a qualitative identification. Analysis by gel electrophoresis indicated that the extracts of meat autoclaved for 15 min contain DNA fragments of 100-300 bp (not shown). The signal of acid treated meat (Fig.4) indicates that the DNA in meat is not completely hydrolysed by acid. Consequently the test is not disturbed by acid treating. Presumably the acid does not reach the nuclear contents under the used conditions.

The method presented here and presence of species-specific stDNA in the genomes of virtually all eukaryotes may allow the development of oligonucleotide probes for the detection of any other species.

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Legends to the figures.

Table I: Probes used in this study.

Fig. 1. Hybridizations of the HMSR (left) and the GMRS probe (right) to 2 µl DNA extracted from horse and chicken meat and to DNA standards. The standards consists of known amounts of genomic DNA purified from horse and chicken blood as described by Ciulla *et al.*, 1988).

Fig. 2. Hybridizations of the HMSR, SSAS, GMRS and MMRS probes to DNA extracts of meat of horse, pig, chicken and turkey.

Fig. 3. Hybridizations of the GMRS and the MMRS probes to varying mixtures of chicken and turkey meat.

Fig. 4. Hybridizations of the MMRS probe to DNA extracts of turkey meat samples autoclaved at 120 °C for varying times and turkey meat incubated in 5 % HOAc.

Fig. 5. Hybridizations of the SSAS, GMRS and HMSR probes to DNA extracts of lean smoked bacon, sausage and luncheon meat.