Journal of Industrial Microbiology & Biotechnology (1998) 21, 115–120

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Species identification in meat by using PCR-generated satellite probes

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A convenient DNA-based identification system is described for testing the species origin of meat samples. Probes are generated by PCR with primers binding to species-specific satellite DNA and hybridized to DNA purified from meat. This method is more robust and versatile than methods based on oligonucleotide hybridization. With the exception of a slight cross-reaction of mutton and beef, each probe only recognized the species from which it was derived. Purifying the DNA with a DNA-binding resin improved the sensitivity. Admixtures of 0.1–0.5% can be detected in raw meat and 0.5–5% in autoclaved meat samples. The method can be adapted to detect any eukaryotic species for which species-specific DNA sequences are available. This method has proven its value in the routine inspection of meat samples by revealing more cases of deliberate or accidental species substitution and admixture than conventional techniques.

Keywords: species identification; DNA isolation; meat testing; satellite DNA

Introduction

Testing the species origin of meat samples is relevant for economic, religious and public-health reasons. In addition, wildlife management often requires identification of the animals' remains resulting from poaching. Conventional techniques are based on the electrophoretic or immunochemical detection of species-specific proteins.

Recently, methods have been developed that are based on the hybridization of specific DNA probes. Several authors [1,4,11,15,16,38] described the identification of species in cooked or autoclaved meat samples by genomic DNA probes. Discrimination of related species, like cattle and sheep remained difficult. Buntjer *et al* [5] and Hunt *et al* [19] introduced the use of satellite-specific probes. DNA satellites are abundant tandem repeats that in mammals occupy up to 20% of the genome and have species-specific sequences as a result of a concerted evolution. Oligonucleotides designed on the basis of satellites allowed not only the detection of processed or autoclaved meat samples, but also discriminated between closely related species. However, the sensitivity of oligonucleotide hybridization to small variations in temperature, salt concentration and other experimental conditions [32] makes it difficult to standardize.

Here we describe a DNA-based species identification test based on probes that are generated by PCR amplification of segments of satellite DNA. This results in a robust procedure that is compatible with any labeling and detection technique. The performance of this method, using digoxigenin labeling with test samples is described. The method has proven to be practical and reliable during routine inspection of meat samples.

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Received 12 January 1998; accepted 15 June 1998

Materials and methods

DNA extraction

Meat samples (5 g) were homogenized for 1 min in a Waring blender in 20 ml 0.4 M NaCl, 10 mM Tris/HCl (pH 8.0), 1 mM EDTA. After adding 50 μl 10% SDS and 125 μl proteinase K (1 mg ml^{-1}) to 750 μl homogenate, samples were digested overnight at 65°C. NaCl/EtOH purification [30] of the DNA was carried out by adding 250 μl saturated NaCl, centrifuging the mixture for 15 min at 10 000 $\times$ *g*, adding 1 ml cold (–20°C) ethanol to 500 μl supernatant, centrifuging the mixture again and dissolving the dried pellet in 50 μl 10 mM Tris/HCl (pH 8.0) containing 1 mM EDTA. Alternatively, after digestion and centrifugation, DNA was purified by binding it to and elution from a Wizard column following the instructions of the manufacturer (Promega, Madison, WI, USA). DNA concentrations were about 1 μg μl^{-1} after NaCl/EtOH precipitation and 0.2–0.5 μg μl^{-1} after Wizard purification as measured by absorption at 260 and 280 nm.

Test samples

Test samples of beef with combined admixtures of 0.1% (w/w) were prepared by mixing chopped beef (to 100%) with 0.1% chicken, 0.1% turkey, 0.1% pork, 0.1% horse as well as 0.1% mutton, 2% (w/w) NaCl/$NaNO_2$ (167:1, w/w), 5% (w/w) starch, 0.05% (w/w) ascorbic acid and 9.25% (w/w) water. Samples of beef with combined mixtures of 0.5%, 1%, 5% or 10%, w/w, respectively, of the five species were prepared likewise. Test samples of chopped pork with admixture of beef were prepared by mixing 0.1%, 0.5%, 1%, 5%, 10% (w/w), respectively, of beef with 2% NaCl/$NaNO_2$, 5% (w/w) starch, 0.05% (w/w) ascorbic acid,

9.25% (w/w) water and pork to 100%. Test samples of beef with admixture of lard were prepared by mixing 0.1%, 0.5%, 1%, 5%, 10% (w/w), respectively, of lard with 2% $NaCl/NaNO_2$, 5% (w/w) starch, 0.05% (w/w) ascorbic acid, 9.25% (w/w) water and beef to 100%. Samples were divided in aliquots of 5 g, pressed to slabs of about 1 mm thickness, sealed in plastic foil and, if indicated, heated for 5, 10 or 45 min at 100°C or for 10 min at 115°C.

Amplification of species-specific probes

Probes were generated and labeled in 100 μl containing 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 1.5 mM $MgCl_2$, 2 μg ml^{-1} BSA, 200 μM dATP, dCTP and dGTP, 190 μM dTTP, 10 μM digoxigenin-dTTP (Boehringer, Mannheim, Germany), 0.5–1 μM of both primers (Table 1), 0.5 ng genomic DNA and 2 U of Amplitaq DNA polymerase (Perkin Elmer, Foster City, CA, USA) with 25–30 cycles of 1 min at 92°C, 2 min at the indicated annealing temperature and 2 min at 72°C.

Hybridization

After heating DNA solutions at 95°C for 5 min and cooling on ice, 2 μl was spotted on a positively charged nylon membrane (Boehringer). DNA was fixed on the blots by UV irradiation for 5 min and prehybridized for 2 h at 42°C in 50% (v/v) formamide, $5 \times$ SSC ($1 \times$ SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 50 mM sodium phosphate (pH 7.0), 5 mM EDTA, $5 \times$ Denhardt's solution [32], 0.25 mg ml^{-1} herring sperm DNA (added fresh and boiled just before use). Hybridization was overnight at 42°C DNA in 25 ml per 100 cm^2 membrane prehybridization mix containing about 200 ng ml^{-1} probe (200 μl PCR reaction mixture per 10 ml hybridization mix), boiled and kept on ice just before use. Blots were washed 2×5 min at room temperature in $2 \times$ SSC, 0.1% SDS and 2×15 min at 42°C in $0.1 \times$ SSC.

Detection of the probe

Binding of the probe to the membrane was detected by consecutive incubations at room temperature in 100 mM Tris/HCl (pH 7.5), 150 mM NaCl (5 min), 50 mg ml^{-1} sodium caseinate in the same Tris/HCl/NaCl buffer (30 min), Tris/HCl/NaCl (5 min), 0.15 U ml^{-1} of a conjugate of anti-digoxigenine-IgG and alkaline phosphatase (Boehringer, 1: 5000) in Tris/HCl/NaCl, 20 ml per 100 cm^2 membrane (30 min), Tris/HCl/NaCl (5 min) and finally in freshly prepared staining solution (0.5 mg ml^{-1} Fast-Violet B (Serva, Heidelberg, Germany) in 100 mM Tris/HCl (pH 8.2), 100 mM NaCl, 50 mM $MgCl_2$ with 0.05 vol 1% (w/v) naphthol AS-E phosphate (Serva) in DMSO) until the background of the staining appeared. Staining was stopped by washing the membrane in H_2O.

Results and discussion

DNA isolation

Figure 1 shows the patterns after purification of DNA by salting-out [30] and the Wizard procedure, respectively. Clearly, specific binding to a resin as in the Wizard columns removed material with low molecular weight that is retained by the salting-out procedure. Although the species origin can also be determined by hybridization to crude DNA preparations (see below and [5]), the convenient Wizard purification gave the most even binding of the DNA to the filter membrane and a slightly more sensitive detection of admixtures (see below).

Specificity

As shown in Figure 2a, probes generated with the primers listed in Table 1 only recognized the species from which the probes were derived except the cross-reacting beef and mutton probes. This cross-specificity only impeded the detection of low levels of admixtures of mutton in beef or vice versa (see below).

The probes used in this study will not discriminate between closely related species that have similar satellites, like goat and sheep, or common cattle, bison and water buffalo [21]. If relevant, these species can easily be discriminated by restriction-enzyme digestion of PCR-generated satellite fragments [11,21, unpublished results].

Sensitivity

Figure 2b shows a typical result of salted-out and Wizard-purified test samples hybridized to the horse probe. This probe detected admixtures of horse in beef of 0.1% after cooking for up to 45 min and 0.5% after autoclaving at 115°C for 10 min. Similar levels of admixtures were

Table 1 Primers for the generation of species-specific satellite probes

Species-specific repeat	Literature and Genbank reference	Primers (5′ → 3′)	Annealing temperature	Length of PCR product
Chicken CNM satellite	[27], X51431	gcgttttctcttcgcaaatcc acgcgtgattttcgcttaaatg	55°C	50-bp multimers
Turkey TM satellite	[28], X66696	gtatttgtgggagaaaaaggg cacaaatacctgtttttacacg	55°C	50-bp multimers
Horse major satellite	[37], X70916	ttctgctctgggtgtgctactt ctacttcagccagatcaggc	55°C	221-bp multimers
Cattle satellite IV	[21,35], X00979	aagcttgtgacagatagaacgat caagctgtctagaattcaggga	55°C	603 bp
Sheep satellite I	[11,31], X01839	gttaggtgtaattagcctcgcgagaa aagcatgacattgctgctaagttc	60°C	374 bp
Pig Ac2 satellite	[20], X51561-51565	ggagcgtggcccaatgca attgaatccactgcattcaatc	55°C	≥100 bp

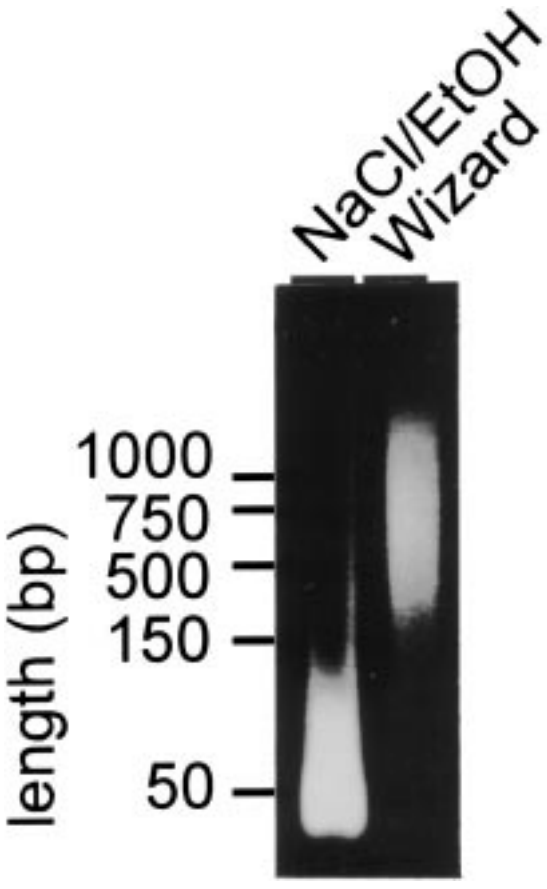

Figure 1 Agarose gel electrophoresis of DNA isolated from uncooked meat by salting-out (NaCl/EtOH) and Wizard purification. Heating the meat gave similar results, but decreased the yield of the DNA.

detected by other probes (Table 2). With mutton, lard and beef, the sensitivity was improved by Wizard purification of the DNA. However, the cross-reaction of the mutton probe with cattle limited the detection to levels of 0.5% in raw meat and 5% in heated meat. Further, the detection of lard, which contains more fat and less DNA than muscle tissue, is less sensitive than the detection of pork. However, in all cases the sensitivity is adequate for routine inspection of meat samples.

Adaptations of test

In this study the probes were labeled with digoxigenin group, which was detected by an immunochemical procedure with a chromogenic alkaline-phosphatase substrate. The method can be easily adapted to the more sensitive chemoluminescent substrates [5,19] or to any radioactive or nonradioactive labelling and detection technique, including the direct conjugation of DNA to alkaline phosphatase (Amersham, Little Chalfont, Bucks, UK). As an option, the PCR with the primers listed in Table 1 may be carried out on the sample DNA as template with real-time quantitation of the amplification products by the 5′ nuclease assay [24,39].

Table 3 lists repetitive elements of other species that are used as a source of meat on which species-specific tests may be based, including repeats specific for groups of species (cetaceans, ruminants, deer) and for bovid males (bulls,

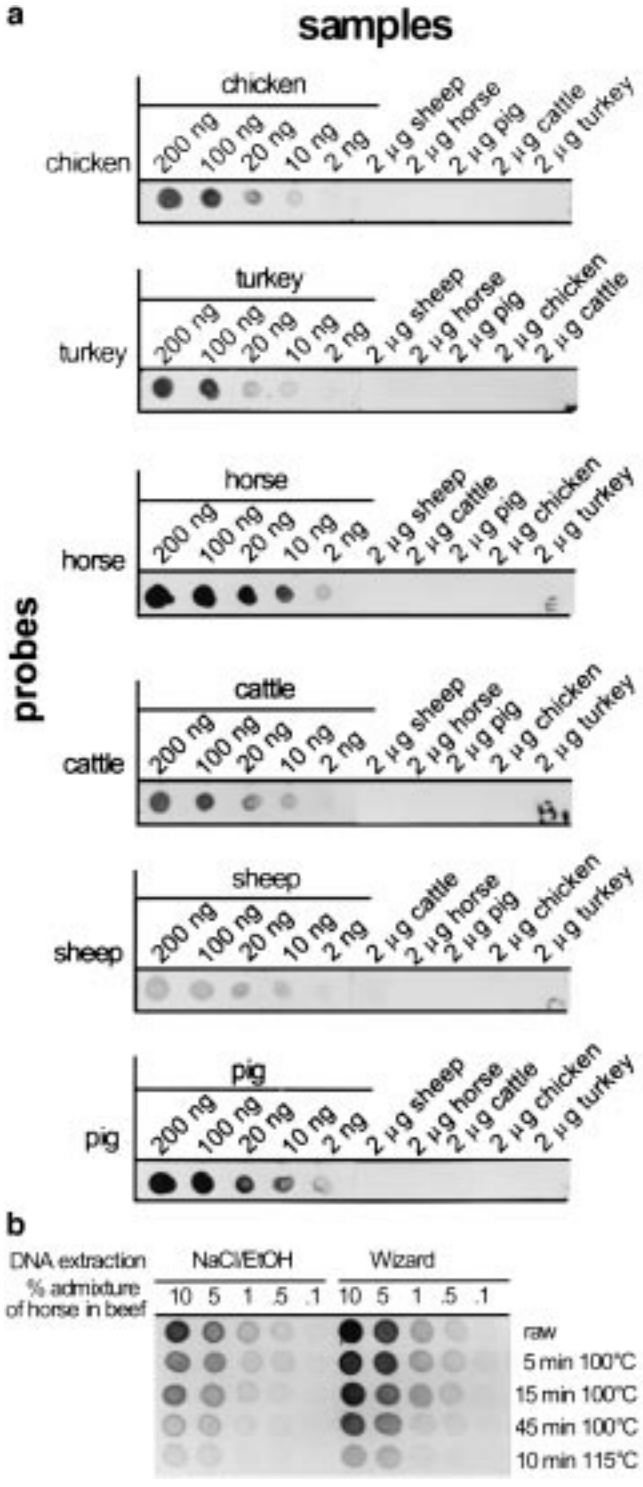

Figure 2 Immunochemical detection of the binding of probes to DNA from meat extracts. (a) Specific binding of probes to DNA from the corresponding species, isolated by Wizard purification. The same specificity was observed with DNA purified by the NaCl/EtOH method (not shown). (b) Detection of admixture of horse meat in beef subjected to the indicated heat treatment. DNA was isolated by the NaCl/EtOH or the Wizard protocol as indicated. The amount of DNA spotted corresponds to extracts of 3.2 mg meat. Reproduction has decreased the contrast relative to the original blot, on which clear signals of the sheep probe hybridizing to 2 μg cattle DNA and of the cattle probe hybridizing to 2 μg sheep DNA could be detected.

Table 2 Percentages of admixtures that can be detected after NaCl/ethanol precipitation (N) or Wizard purification (W) of the DNA. Admixtures of chicken, turkey, mutton, horse and pork were tested in samples in which combined admixtures of all five species had been added to beef. Admixture of beef was tested in samples in which beef had been added to pork. Admixture of lard was tested in samples in which lard had been added to beef

	Raw		5 min 100°C		15 min 100°C		45 min 100°C		10 min 115°C	
	N	W	N	W	N	W	N	W	N	W
Chicken	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Turkey	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	1
Beef[a]	1	0.5	1	0.5	5	1	5	1	5	5
Mutton[b]	1	0.5	5	1	5	1	5	5	5	5
Horse	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.5	0.5
Pork	0.1	0.1	0.1	0.1	0.5	0.5	0.5	0.5	0.5	0.5
Lard	0.5	0.5	5	0.5	nd	0.5	5	0.5	5	5

[a]Since the cattle probe cross-reacted with beef, detection of beef admixture in mutton is probably less sensitive.
[b]Since the sheep probe cross-reacted with cattle, lower levels of admixtures may be detected in meat of other species.
nd, not determined.

rams). However, the specificity and sensitivity of probes based on these satellites should be verified if the probes are used for the purpose of meat inspection.

Comparison with other methods

The main advantage of all DNA-based methods is the combination of a high specificity and the ability to test highly processed samples [1–5,9–11,14,19]. However, samples in which the DNA has been degraded by extreme heating may still be analyzed by the less specific protein-based methods. The main advantage of the method described here relative to methods based on oligonucleotide probes [5,19] is that the hybridization to the longer, PCR-generated probes is not affected by small variations in temperature, incubation time and composition of the buffer during hybridization and washing [32]. Indeed we obtained similar results after washing with a $1 \times$ SSC instead of $0.1 \times$ SSC buffer.

Other DNA species-identification methods are not based on hybridization to specific probes but on the generation via PCR of species-specific DNA fingerprints [9], mitochondrial sequences [3,17] or mitochondrial RFLP patterns [29]. These methods are especially useful for exotic species and if there is no prior information on the suspected species origin, but are less or not suitable for the detection of an admixture. Admixtures may be determined by a convenient PCR-RFLP procedure of mitochondrial DNA, but this method is less suitable than hybridization assays for testing many samples.

Results of routine screening

The procedure described in this paper has proven suitable for routine inspection of meat samples in the Netherlands. As an example, Figure 3 shows that from 20 randomly sampled hamburgers, several contained pork, horse or chicken instead of or added to the beef. In other cases of undeclared substitutions and admixtures we found turkey in chicken products, substitution of horse meat for beef in croquettes, horse meat and pork in beef salad, mutton in minced veal, or pork in beef products to be exported to Islamic countries. While substitution may be assumed to be fraudulent, admixture often appeared to be accidental and to result from inadequate cleaning of equipment used consecutively for different species. In these cases the DNA hybridization tests have led to improvements in production procedures.

Table 3 Species-specific satellite probes of other species

Species-specific repeat	Literature and Genbank reference	Primers (5′ → 3′)	Annealing temperature	Length of PCR product
Waterfowl RBMII satellite	[25, unpublished], X61401-X61429	gactgaaaactcggcccacac gctggctagggttatatttagg	55°C	178 bp
Toulouse goose TGI satellite	[40, unpublished]	tggactctctggactcactgc ctggcaccactggggatgcag	55°C	43 bp
Ostrich *Alu*I satellite	[8], AJ001419			
Ruminant BovA SINE	[6, 23], X64126	aatggcaacccactccagta ctcagtcgtgtccgactctt	55°C	101 bp
Ruminant BovB SINE	[6, 23], X64125	gtcatgtatggatgtgagagt tcagggtcttttccaatgagt	55°C	247 bp
Cattle and sheep male BRY-1 repeat	[2,26,34], X74507	ggatccgagacacagaacaggctgc ttgatcaagctaatccatccatcctat	56°C	309 bp
Deer CcsatI	[22,33]	tgcagagcaattccttgttgc tcagggtccctctcacatac	55°C	300–500 bp
Roe deer CcsatIII	[7], Y10686	ccctcgctctccaatgaagc cgaggactccattttctgaatg	55°	2244 bp
Pronghorn satellite I	[12], U03038			
Primate alphoid satellite	[8, 36]	atacacacaacaaggaagttac tcaactcacagagttgaacgatc	52°C	
Cetacean satellite	[18]			
Dog CFA-SAT satellite	[16]			
Rabbit 354-bp *Hin*dIII satellite	[13]			

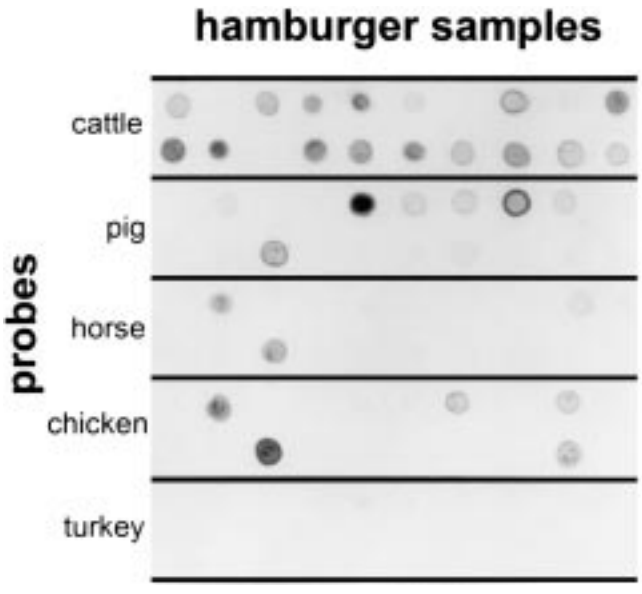

Figure 3 Routine survey of 20 hamburger samples with probes specific for cattle, pig, horse, chicken and turkey, respectively.

Acknowledgements

This research was supported by the General Inspectorate of Health Protection, Rijswijk. The systematic study of the use of satellite probes for species identification was initiated by the late Dr N Haagsma (Utrecht University). We thank Drs A Ruiter (Utrecht University), D Groothuis (Inspection of Health Protection, Rijswijk) and G Wijngaards (TNO, Zeist) for useful discussions.

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