FOOD COMPOSITION AND ADDITIVES

Rapid Visual Detection of Eight Meat Species Using Optical Thin-Film Biosensor Chips

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Adulteration of meat products has become a very serious issue nowadays. To protect consumer rights, food labeling is required in many countries, and efficient and accurate detection methods are essential as well. This paper reports an innovative method for the rapid detection and identification of meat species based on a silicon-based optical thin-film biosensor chip with which color change results can be perceived by the naked eye without any expensive instruments. This biosensor system can simultaneously and specifically detect eight meat species, including deer, rabbit, duck, chicken, beef, horse, sheep, and pork. The absolute detection limit of this method was 0.5 pg of deer/beef DNA, and the practical detection limit was 0.001%. The biosensor detection can be completed within 30 min after PCR amplification. Therefore, this assay permits specific, sensitive, rapid, and simple detection of meat species in raw or cooked meat products.

Authentication and traceability of meat and meat products have become a concern for governments, consumers, and food industries (1). To avoid unfair market competition and to protect consumers from falsely labeled meat products, it is imperative to provide reliable and effective methods of analysis that facilitate routine control tests of meat species in different foods and feedstuffs. Currently, numerous analytical methods are based on identification of species-specific proteins by means of electrophoretic and/or immunological assays (2–5). However, these techniques are not reliable for identifying species in heated or baked products as a result of denaturation and degradation of the proteins (6–8). More recently, DNA-based assays as an indirect approach have also been used for the accurate detection of the animal species due to its higher stability than the proteins (9–11). Because there are different species in meat foods and/or different components in processed food, it is necessary to establish multiple detection assays. Among them, the microarray method has high throughput for simultaneous identification of multiple proteins or DNA targets (12–14). Optical thin-film biosensor chips, one of the microarray methods, have been mainly applied to the detection of foodborne pathogens and genetically modified organisms (15–18). Compared with regular biochips, the detection procedure of optical thin-film biosensor chips is simple and convenient; only heat denaturization plus a few minutes of hybridization steps are needed. Moreover, experimental results are visible to the naked eye without any specific instruments because the biotinylated amplification products combining with aldehyde-labeled probes deposited on the thin-film surface by enzymatic catalysis alter the wavelength of light reflected by the optical layer and result in a perceived color change on the surface (gold to blue-purple; 19).

In this study, we developed an optical thin-film microarray method on a silicon-based surface for simultaneously identifying eight meat species, including deer, rabbit, duck, chicken, beef, horse, sheep, and pork. In general, this method reduces the need for costly sophisticated equipment, exhibits excellent sensitivity and specificity, and is very competitive among existing technologies.

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Experimental

Samples

All muscle tissue samples from deer, rabbit, duck, chicken, beef, horse, sheep, and pork were purchased from a local supermarket in Nanjing, China. Meat samples were stored at –20°C until analysis.

DNA Extraction

DNA extraction was performed using a modified cetyl trimethylammonium bromide (CTAB) method (20). The samples of meat were ground to a fine powder with a SPEX 6850 freezer mill (SPEX, Metuchen, NJ) and then were baked at 60°C for 3 h. From the resulting homogenized sample, all DNA extractions were done in duplicate. Two portions of 100 mg each of the homogenized sample were used for DNA extraction. Clean instruments were used for each sample to prevent cross-contamination. Each sample was mixed with 1.5 mL CTAB extraction buffer (20 g/L CTAB, 1.4 M NaCl, 0.1 M [tris(hydroxymethyl) aminomethane (Tris)]/HCl, 20 mmol/L Na_2EDTA, pH 8.0) and 10 μL Proteinase K solution (20 mg/mL; Qiagen, Hilden, Germany) in a 2 mL

Table 1. Sequences of PCR primers and capture probes for the detection of eight meat species

Species	Target gene	Sequence	Size, bp	Accession No.	Ref.
Deer	cytb	F[a]: 5′ATCATCGCAGCACTCGCTATAGTACACT3′ R[b]: 5′biotin- ATCTCCAAGCAGGTCTGGTGCGAATAATA3′ P[c]: 5′ALD[d]-aaaaaaaaaaGGGTATCTTACTTCTAATACTCTTCCTAAT3′	195	KC509614	This study
Rabbit	cytb	F: 5′GTCTTAATTCACCTCCTCTTTC3′ R: 5′biotin-GGAGAAGAATGGCTACAAGGAAA3′ P: 5′ALD-aaaaaaaaaaAGGAATTCCTTCAAACTCACATAA AAT3′	139	HG810791	This study
Duck	mtDNA D-loop	F: 5′TCCACCACCTCAATGCGTAATCGCG3′ R: 5′biotin-CCCGAAGTC ACTGGGAAGGGCCAG3′ P: 5′ALD-aaaaaaaaaaGCGCCTCTGGTTCCTTTTATTTTTTCCGG3′	109	JQ237612	This study
Chicken	mtDNA	F: 5′CCCTCCTCCTTTCATCCTCAT3′ R: 5′biotin-GTCATAGCGGAACCGTGGATA3′ P: 5′ALD-aaaaaaaaaaCTATGAATCCGGGCCTC3′	62	KF826490	21
Beef	mtDNA	F: 5′GCCATATACTCTCCTTGGTGACA3′ R: 5′biotin-GTAGGCTTGGGAATAGTACGA3′ P: 5′ALD-aaaaaaaaaaCACAACTTTTATCACAATCCAGAACTGACACCAAC3′	271	KF926377	22
Horse	cytb	F: 5′ACA ACGAAGCATAATATTCCGGCCTCT3′ R: 5′biotin-CAGTTGGCCGATAATTACGTATGGGT3′ P: 5′ALD-aaaaaaaaaaGACTCTTAGTGGCAGACTTACTGACACTAACAT3′	127	JF511459	This study
Sheep	cytb	F: 5′TATTACACCATTAAAGACATCCTAGGT3′ R: 5′biotin-GGTCTCCGAGTAAGTCAGGC3′ P: 5′ALD-aaaaaaaaaaTACTAATCCTCATCCTCATGCTA3′	91	KF677297	This study
Pork	cytb	F: 5′TTGCA AATCCTAACAGGCCTG3′ R: 5′biotin-GTA ACTGATGAGAAAGCTGT3′ P: 5′ALD-aaaaaaaaaaTAGCAATACATTACACATCAGACACA3′	74	KF799992	This study

[a] Forward PCR primer.

[b] Reverse PCR primer.

[c] Probe.

[d] ALD = Aldehyde modification.

tube and then were incubated for 1 h at 65°C under permanent horizontal vibration using Thermomixer® Comfort (Eppendorf, Hamburg, Germany). Samples were centrifuged (Beckman Coulter, Krefeld, Germany) for 10 min at 13 000 × *g*; the supernatant was transferred by pipet to a new 2 mL tube, mixed with 750 µL chloroform, vortexed, (Ika Vortex Genius 3; Staufen, Germany) and centrifuged again for 5 min at 13 000 × *g*. The upper phase was transferred into a new 2 mL tube, and the volume of the solution was determined. Two volumes of precipitation buffer (0.5% CTAB, 0.04 M NaCl, pH 8.0) were added and incubated for 60 min at room temperature without agitation. The samples were centrifuged for 15 min at 13 000 × *g*, the supernatant was discarded, the pellet was resuspended in 350 µL NaCl solution (1.2 M), vortexed, transferred into a new 2 mL tube, and mixed with 350 µL chloroform. After centrifugation for 10 min at 13 000 × *g*, the upper phase was transferred into a new 1.5 mL tube; 0.8 volume isopropanol was added for nucleic acid precipitation. After incubation at room temperature for 20 min, the samples were centrifuged at 13 000 × g for 10 min, and the supernatant was discarded. The pellet was washed with 500 µL 70% ethanol and redissolved in 100 µL TE buffer [10 mmol/L Tris, 1 mmol/L Na_2EDTA, pH 8.0] for further use. The DNA was quantified with a DU 640 spectrophotometer (Beckman Coulter) and diluted to 20 ng/µL. DNA ladder marker (50 bp; Takara Bio Inc., Otsu, Japan) was used for calibration.

Primer and Probe Design and Synthesis

All DNA oligonucleotides were synthesized by Invitrogen Co. (Shanghai, China). The primers and probes were established in this work or were taken from published single PCR systems (for details, *see* Table 1). To increase the specificity and sensitivity of the method, the sequences of primers and probes of mitochondrial DNA (mtDNA) D-loop gene from duck and cytochrome b (cytb) gene from deer, rabbit, horse, sheep, and pork were designed according to the optimum principle of primer and probe design using Oligo 6 Demo software (Oligo, http://www.oligo.net). During the design, all primers and probes were successfully checked for relevant homologies by Basic Local Alignment Search Tool non-redundant (BLAST nr) database search within GenBank databases (National Center for Biotechnology Information, Bethesda, MD). The reverse primers for PCR were synthesized with a biotin modification at their 5′ ends for detection. The probes have 10 deoxyadenosine residues that constitute a "spacer" with an aldehyde group modification at their 5′ ends for conjugating to amino groups on the chip surface, followed by about 30 nucleotides complementary to the corresponding target sequence.

PCR Amplification

PCR reactions were carried out in 25 µL reaction mixtures containing 1× PCR buffer (Qiagen), 2 mmol/L $MgCl_2$, 0.1 mmol/L deoxyribonucleoside triphosphates, 0.2 µmol/L of each primer, 1 U HotMaster *Taq* DNA polymerase (Qiagen), and 5 µL template DNA on an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) as follows: initial step of 15 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, and one step of 5 min at 72°C. The PCR products were analyzed by electrophoresis on 3% (w/v) agarose gels (Takara Bio), stained

with ethidium bromide (EB; Takara Bio), and photographed using a GelDocXR system (Biorad, Hercules, CA).

Preparation of the Optical Thin-Film Biosensor Chips

The biosensor chips were obtained from Biostar Microtech Corp. (City of Industry, CA) and cut into 7×7 mm rectangles by a laser (Lajamin Laser, Beijing, China). The biosensors were prepared following the procedure described by Zhong et al. (19).

Standard Assay Protocol

The biosensor chips were loaded with the aldehyde-labeled probes by spotting (50 nL/spot) a 1.0 μmol/L probe solution (in 0.1 M sodium phosphate buffer, pH 7.8) via an AD3200 (Biodot, Irvine, CA). The chips were allowed to sit at room temperature and 70% humidity for 3 h and then were rinsed with 0.1% sodium dodecyl sulfate. The PCR amplification targets were then hybridized with probes on the chip. The PCR products were denatured at 95°C for 3 min and hybridized on the chip for 5 min at 45°C in the hybridization buffer [5 × standard saline citrate (SSC) and 5 mg/mL acid-treated casein (ATC)]. After being rinsed three times with 0.1× SSC, the chips were incubated with an anti-biotin IgG-horseradish peroxidase conjugate (Jackson ImmunoResearch, West Grove, PA) 1:1000 dilution from a 1 mg/mL stock in a buffer containing 5× SSC, 5 mg/mL ATC, and 10% glycerol for 5 min in hybridization buffer. After rinsing three times with 0.1 × SSC, 100 μL tetramethylbenzidine was added and incubated for 5 min at room temperature in the dark. The chips were then rinsed with double distilled (dd) H_2O, air-dried, and visually observed with the naked eye. The chips can also be imaged with a dissection microscope with a digital camera.

Sensitivity Detection Assay

To verify the sensitivity of the biosensor chips for detecting meat species, deer and beef meat samples were randomly chosen as test samples. Deer and beef genomic DNA was quantified by UV spectrometry. To determine the absolute LOD, five levels of serial dilution containing 1, 0.1, 0.01, 0.001, and 0.0001 ng deer and beef genomic DNA/μL were prepared with ddH_2O. A 5 μL volume of diluted sample was used for PCR, so that the amount of deer/beef genomic DNA in the final 25 μL was 5, 0.5, 0.05, 0.005, and 0.0005 ng, respectively. A series of mixtures with different percentages of deer/beef with pork powder, i.e., 10, 1, 0.1, 0.01, and 0.001% (w/w), were used to determine the relative LOD and prepared as follows. A mixture with 10% (w/w) deer/beef content was made by mixing 1 g deer/beef powder with 9 g pork powder; then, mixtures with 1% (w/w) deer/beef content were made by mixing 1 g mixture with 10% (w/w) deer/beef content with 9 g of pork powder. In a similar manner, mixtures with 0.1, 0.01, and 0.001% (w/w) deer/beef content were prepared. DNA extraction was performed according to the CTAB protocol (20) and detection by the thin-film biosensor chip assay as described above.

Analysis of Retail Samples

Five food samples of different product groups were obtained from local markets in Nanjing, China, including mutton rolls (with mutton declared on the label), pork floss (with pork declared on the label), pork sausage (with pork declared on the label), dried beef cubes (with beef declared on the label), and spiced rabbit (with rabbit declared on the label). DNA extraction of these five samples was performed according to the CTAB protocol (20), and extracts were amplified by PCR and analyzed by the biosensor chips assay mentioned above.

Results and Discussion

Amplification of Eight Target Fragments by PCR

The PCR products for the eight species are shown in Figure 1. The sizes of amplified fragments were the same as predicted (Table 1). The results also showed that no EB staining was observed in the blank lane, indicating that no contamination occurred during PCR. From lanes 1–8, only one clear-cut band with intensive staining and molecular size as expected was found for each species, indicating that the eight PCR systems were specific and accurate.

Detection of Eight Meat Species with Biosensor Chips

The biosensor chips spotted by robotic pipetting (50 nL/spot) were used to detect eight meat species (Figure 2A). M represented the positive control biotin-dA20 (5′-ALD-AAAAAAAAAAAAAAAAAAAA-3′-biotin) that always exhibited signals if the chip detection system worked. In this system, all other species were negative controls for the detected species. Results showed that specific signals were detected from eight meat species (Figure 2B). For example, ddH_2O instead of PCR-amplified products was used for hybridization in a 100 μL reaction, and the assay showed that only positive signals were observed. The DNA targets amplified from deer were used for hybridization to the biosensor chip, and the assay resulted in one set of colored dots. The other seven DNA targets also showed their own specific sets of colored dots (Figure 2B). No false-positive signals were observed among these tests. Our data indicate that this chip can be considered an effective and specific analytical tool to detect the presence of these eight meat species, and it may be further modified to accommodate all needs to detect commercialized meat products.

Sensitivity and Limitations of Biosensor Chips

Five concentration gradients of deer and beef genomic DNA in serial diluted samples were applied to the confirmation of the absolute LOD of the chips. Chips were spotted by robotic pipetting as shown in Figure 2A. The results showed that all five levels produced the hybridization signals but not the blank control, and the changes in hybridization signals were consistent with the decrease in deer and beef genomic DNA concentration; when deer and beef genomic DNA content was reduced to 0.5 pg, weak signals could also be observed (Figure 3A), which indicated that the absolute LOD of the biosensor chips was 0.5 pg. The practical LOD of the biosensor chips was determined by hybridizing different percentages of deer/beef mixtures, including 10, 1, 0.1, 0.01, and 0.001% (w/w) deer/beef. As indicated in Figure 3B, a hybridization signal could be observed from blended samples with deer or beef concentration down to 0.001%, which indicates that the practical LOD of this method

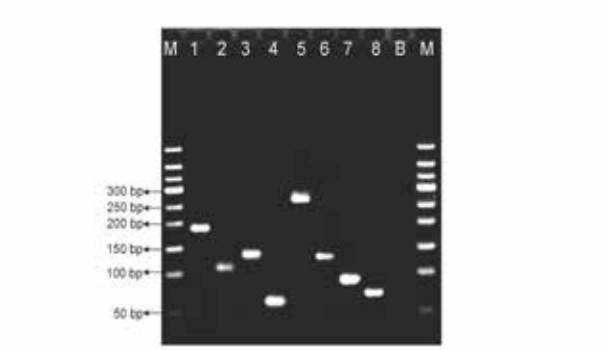

Figure 1. PCR amplification of target DNA fragments from eight meat species. Lanes: M, 50 bp DNA ladder marker; 1, deer (195 bp); 2, rabbit (139 bp); 3, duck (109 bp); 4, chicken (62 bp); 5, beef (271 bp); 6, horse (127 bp); 7, sheep (91 bp); 8, pork (74 bp); B, blank control (ddH_2O).

was 0.001%. Because of differences in primers and probes, the biosensor chip had a much greater sensitivity than that reported earlier (23, 24). Therefore, the sensitivity of the chip in our study should be sufficient to satisfy the requirements for practical use.

Detection of Eight Meat Species in Retail Samples by Biosensor Chips

To confirm the accuracy and reliability of biosensor chips, five different retail samples were chosen as test samples. Chips were spotted by robotic pipetting as shown in Figure 2A. The DNA targets amplified by PCR were hybridized to the chips to analyze the eight meat species in retail samples. The detection results showed that all positive controls underwent color reactions, which ensured that the biosensor chip detection had proceeded normally and excluded the possibility of false negatives; only pork floss and spiced rabbit of the five samples were consistent with product components declared on the label (Figure 4). This technique would be useful for effective control of the adulterated meat products and the labeling violation requirements for meat and meat products.

Conclusions

Species identification and authentication of meat and meat products is important and necessary because of health, religious, and economic reasons (25). Many different assays and strategies are available for tracing meat adulteration and differentiating species present in mixed meat. However, in order to meet the needs of large scale and simple application, the development of optical thin-film biosensor chip assays has been presented. This technique meets the needs of rapid, simple, and high throughput detection with little reagent consumption. Furthermore, the method offers simple visible detection with the naked eye without any expensive instruments. Therefore, this chip can be recommended as an effective monitoring and assessment tool for species identification, especially for meat traceability and Halal authentication

Acknowledgments

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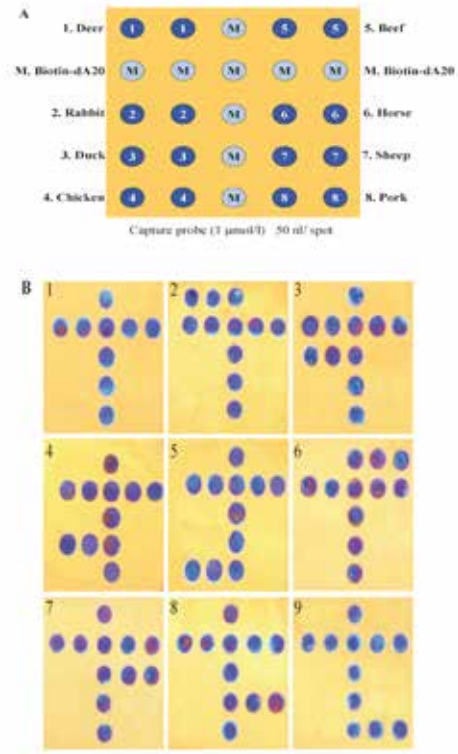

Figure 2. Specificity of meat species detection on thin-film biosensor chips with capture probes spotted by a computer-controlled dispenser. Each spot comprised 50 nL of 1.0 μmol/L probe solution. Capture probes were printed in the order shown in (A). M biotin-dA20 (positive control and marker); 1, cytb gene (deer); 2, cytb gene (rabbit); 3, mtDNA D-loop gene (duck); 4, mtDNA gene (chicken); 5, mtDNA gene (beef); 6, cytb gene (horse); 7, cytb gene (sheep); cytb gene (pork). (B) Detection results of the meat species-specific genes on thin-film biochips: 1, blank control (ddH_2O); 2, deer; 3, rabbit; 4, duck; 5, chicken; 6, beef; 7, horse; 8, sheep; 9, pork.

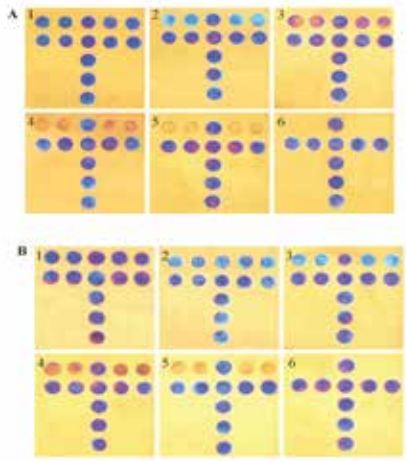

Figure 3. Absolute sensitivity (A) and Practical detection limit (B) of thin-film biosensor chip method for detecting deer and beef. The results of A showed the change of hybridized signals of different deer/beef genomic DNA levels: 1, 5 ng; 2, 0.5 ng; 3, 0.05 ng; 4, 0.005 ng; 5, 0.0005 ng; 6, blank control (ddH_2O), and the results of B indicated the hybridized signals obtained from different concentrations of deer or beef in pork powder: 1, 10%; 2, 1%; 3, 0.1%; 4, 0.01%; 5, 0.001%; 6, blank control (ddH_2O).

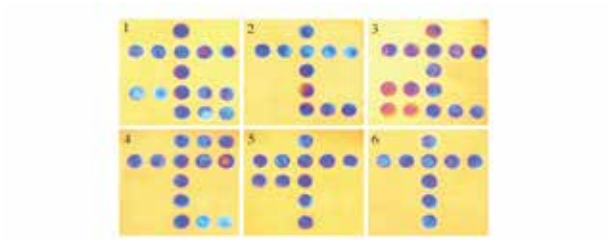

Figure 4. Detection of meat species in retail meat products using the biosensor chip method. 1, Mutton rolls (sheep, pork, and duck showed positive signals); 2, pork floss (pork showed positive signals); 3, pork sausage (pork, chicken, and duck showed positive signals); 4, dried beef cubes (beef and pork showed positive signals); 5, spiced rabbit (rabbit showed positive signals); 6, blank control (ddH_2O).

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