The use of a molecular technique for the detection of porcine ingredients in the Malaysian food market

Abd-ElAziem Farouk, PhD, Mohamed F. Batcha, B.Eng (Biotech), Ralf Greiner, Phd, Hamzah M. Salleh, PhD, Mohamad R. Salleh, PhD, Abdur R. Sirajudin, B.Eng (Biotech).

ABSTRACT

Objective: To develop a molecular technique that is fast and reliable in detecting porcine contamination or ingredients in foods.

Methods: The method applied involved DNA amplification using polymerase chain reaction (PCR) technology. Thus, the sequence of a certain gene found uniquely in pork was identified and its sequence was used to design specific primers for the PCR. The extraction of DNA was optimized in respect to PCR and detection limits were established. The optimized method was then used to identify pork in food products obtained from various local hypermarkets. The latest results were confirmed in triplicates on the 20th April 2006 at the Molecular Biology Laboratory, International Islamic University, Malaysia.

Results: The method was shown to be robust and reliable. Out of 30 food samples not expected to contain pork material, 3 samples were shown to be contaminated with pork material; 2 chocolates and one chicken nugget.

Conclusions: We observed that 2 food products that were labeled as halal showed positive for porcine ingredients, while another that did not have any halal logo but originated from outside Malaysia and exported to many Middle Eastern nations also showed positive.

Saudi Med J 2006; Vol. 27 (9): 1397-1400

Research based on species identification has been pursued since the early stages of DNA sequence analysis. Polymerase chain reaction (PCR)-restriction fragment length polymorphism (PCR-RFLP) method can be used as an analytical and quantitative tool for meat identification. This method uses a specially designed oligonucleotide primer pair to amplify the partial sequences within the 12S ribosomal RNA (12S rRNA) gene of mitochondrial DNA from animals, and has been used successfully to identify porcine, caprine, and bovine meats. However, this pair of primers cannot be utilized to amplify DNA fragment from a dog, cat, fish, duck, goose, turkey, and chicken meat samples. The PCR technique was also applied to identify meat in marinated and heat-treated or fermented products and to the differentiation of closely related species. DNA was isolated from meat samples by using a DNA-binding

From the Biomolecular Engineering Research Group, Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University (Farouk), Biomolecular Engineering Research Group, Department of Biotechnology Engineering, Kulliyyah of Engineering (Salleh HM, Salleh MR, Batcha, Sirajudin), Kuala Lumpur, *Malaysia,* Federal Research Center for Nutrition and Food, Center for Molecular Biology (Greiner), Haid-und-Neu-Strasse, Karlsruhe, *Germany.*

Received 29th January 2006. Accepted for publication in final form 20th May 2006.

Address correspondence and reprint request to: Associate Professor Dr. Abd-ElAziem Farouk, Biomolecular Engineering Research Group, Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, PO Box 10, Kuala Lumpur 50728, *Malaysia.* Tel. +603 (61) 964549. Fax. +603 (61) 964442. E-mail: farouk@iiu.edu.my, aa_farouk@yahoo.com

resin and was subjected to PCR analysis. Primers used were complementary to conserved areas of the vertebrate mitochondrial cytochrome b (cytb) gene and yielded a 35 bp fragment, including a variable 307 bp region. Restriction endonuclease analysis based on sequence data of those fragments was used for differentiation among species. Restriction fragment length polymorphisms (RFLPs) were detected when pig, cattle, wild boar, buffalo, sheep, goat, horse, chicken, and turkey amplicons were cut with AluI, RsaI, TaqI, and HinfI. Analysis of sausages indicates the applicability of this approach to food products containing meat from 3 different species.[1-4]

The halalness of a product be it food, accessories and relationships are important aspect of the Muslim life both spiritually and physically, especially in this time of globalization where a single processed food product can contain ingredients sourced from a dozen nations and its origin not questioned or neglected due to ignorance or lack of technology. Thus, in this era of free trade and mass market globalization, every opportunity is taken by certain food producers to use ingredients that are cheaper and prohibited in certain sections of society due to religious or cultural beliefs and yet not properly or rather accurately labeled. Hence, the field of meat detection especially pork in processed or non-processed food products is hotly pursued by many researchers using various methodologies that are equally effective and reliable in its own way.

The PCR based methodology, which is used in this publication where pig-specific primers were used to amplify a 152bp fragment from the porcine leptin gene (LEP), which is the homologue of murine obese,[5] is a good identification technique as it is PCR-based thus, is most suitable for critical samples in which DNA is largely degraded such as with processed food.

The main objective of the work was to develop a molecular technique that is fast and reliable in detecting porcine contamination or ingredients in foods and to check foods with the Halal label for porcine contamination, which are commercially available on the Malaysian market.

Methods. The meat samples were purchased from nearby hypermarkets. They were divided into 2 categories, processed and non-processed meat consisting of pork for positive control and non-pork meats. Other food products include chocolates, vegetable cakes made of tofu as well as ice-cream.

The DNA isolation. Samples were first carefully proportioned, and to minimize contamination all equipment was washed with 70% ethanol and distilled water before being dried. All tubes and glassware were sterilized by autoclaving including all distilled water. The isolation of DNA from food products, including raw meat, processed meat were performed with the QIAamp® DNA Stool Mini Kit according to the manufacturer instructions (Qiagen, Germany).

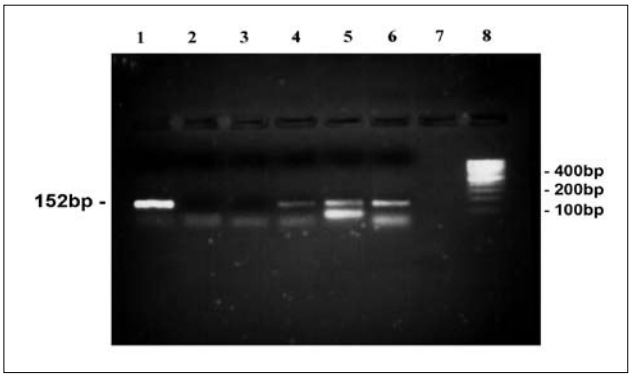

Figure 1 - Gel electrophoresis showing the detection of the specific 152bp leptin gene fragment in processed food product. Lane 1: Pork Sausage, Lane 2: Local Biscuit, Lane 3: Chicken Nugget (Brand A), Lane 4: Chocolate (Brand B) Lane 5: Chocolate (Brand C), Lanes 6: Chicken Nugget (Brand E), Lane 7: empty and Lane 8: 100bp molecular marker.

Samples were suspended in lysis solution followed by the adsorption of impurities by InhibitEX (provided by Qiagen). Then the DNA samples were purified using QIAamp spin columns, which involved the digestion of proteins and binding DNA to the QIAamp silica-gel membrane supplied by Qiagen. After several centrifuge steps, impurities were washed away and finally pure DNA samples were eluted from the spin column. Although the basic steps outlined in the manual (Qiagen) was adhered to, certain modifications with regard to quantity of specimen and thus, reagents were carried out so as to optimize the method to suit our sample materials especially chocolate and ice-cream.

After isolation of the DNA, PCR amplification of the target gene was carried out using various concentrations of DNA.

The PCR Mix. The PCR primers were used to detect the pork leptin gene fragment in food in the PCR amplification. The DNA that was eluted was prepared for PCR amplification using the following protocol. For a 25 μL reaction 1μL of eluted DNA, 2.5 μL 10x PCR buffer containing 1.5mM Mg2+ (Qiagen), 0.25 μL each of forward and reverse primers (20mM), 5 μL 5X Q-Buffer (Qiagen), 15.25 μL PCR water, 0.5 μL dNTPs mix (10mM) and 0.25 μL HotStarTaq DNA polymerase (Qiagen) were used. The forward primer was 5'-TGCAGTCTGTCTCCTCCAAA-3' and the reverse

primers were 5'CGATAATTGGATCACATTTCTG 3'.[1] The PCR cycle was programmed according to the following conditions: 95°C for 15 minutes as HotStarTaq DNA Polymerase by Qiagen is activated by a 15-minutes 95°C incubation step (Qiagen, Hilden, Germany) followed by 29 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute and finally a final elongation of 72ºC for 10 minutes using a MasterCycler Gradient (Eppendorf, Germany). The eluted DNA was diluted with a ratio of 1:10 so as to give a standard total genomic DNA quantity of between 150-200 ng, which was measured using a spectrophotometer at a wavelength of 260nm.

The PCR products were then run in a 1.8% agarose gel in 1X TAE buffer stained with ethidium bromide using Scie-Plas electrophoresis (Scie-Plas, UK) equipment with a voltage of 65V for 2 hours. The results were then visualized and printed using the Alpha Imager gel analysis system (Alpha Innotech Corporation, USA).

Results. The gel electrophoresis suggests that the methodology used to isolate DNA from various samples was reliable and that the specific 152bp porcine leptin gene fragment can be amplified using the specific DNA primers. All types of pork meat including processed meats such as marinated and sausages both imported and local as well as unprocessed pork meat indicated the presence of the 152bp porcine leptin gene fragment.

Having shown that the DNA primers can detect the leptin gene in pork samples the next phase of the work, which is the main aim, was to check the possibility of using the same protocol to detect the presence of porcine DNA contaminants in food products. From the results, the detection of the 152bp porcine leptin gene in lane 1 which is processed pork showed the confirmation of the result. Two chocolate samples (brand B and C) in lanes 4 and 5 and a chicken nugget (brand E) in lane 6 showed the sensitivity of the methodology as well as the uniqueness of the set of primers in detecting pork contamination or ingredients in food samples, whereas lanes 2 and 3 containing other food products did not show any presence of the porcine leptin gene fragment (**Figure 1**).

Discussion. In other methods the use of fluorescence sensor capillary electrophoresis allows the identification of species-specific DNA fingerprints of pork, goat, and beef generated by restriction enzyme digestion by a fluorescence-labeling PCR amplification. The results showed that reliably semi-quantitative levels were below 1% for binary mixtures of pork, goat, and beef. Cooking and autoclaving of meats did not influence the generation of the PCR-RFLP profiles or the analytical accuracy. Highly species-specific primers for pork D-loop mtDNA together with restrictive PCR amplification conditions have greatly influenced the method for detecting a PCR-amplified 531bp band from pork to be more reliable. It has been proved useful for detecting both pork meat and fat in meat mixtures, including those dry-cured and heated by cooking.[6-8]

Recently, a PCR assay has been developed to detect pork in raw and heat-treated meat mixtures. For this purpose, a forward common primer was designed on a conserved DNA sequence in the mitochondrial 12S ribosomal RNA (rRNA) and reverse primers were designed to hybridize on species-specific of pork species DNA sequence. This project is particularly very useful for the accurate detection and identification of species and it helps avoiding mislabeling or fraudulent species substitution in meat mixtures.[9] In another study a rapid high-performance liquid chromatographic procedure for separation of the triacylglycerols (TG) of animal fats using refractive index detection. The TG-profiling of animal fats and fats extracted from genuine meat specimens and from processed fatty foods are of great value for the detection of pork in processed foods samples.[10]

In conclusion, the detection method, which uses minute quantities of DNA as well as resources, has been established at the International Islamic University Malaysia. Thus, so far out of the food samples tested, 3 samples have been tested positive for containing the 152bp fragment of the porcine leptin gene. Thus, this confirms that the food does contain pork ingredients unknowingly as emulsifiers especially in chocolates. Furthermore, as Malaysia is one of the leading exporters of processed food products to Middle Eastern nations, having established a scientific method reinstates the nation's willingness to develop itself as a global halal-hub, as well as to work together in terms of experience and resources with the world community to further develop this field of meat species or ingredient detection in food.

References

1. Meyer R, Hofelein C, Lüthy J, Candrian, U. Polymerase chain reaction-restriction fragment length polymorphism analysis: a simple method for species identification in food. ***J Assoc Off Anal Chem*** 1995; 78: 1542-1551.
2. Meyer R, Candrian U. PCR based analysis for the identification and characterization of food components. ***Lebensm Wiss Technol*** 1996; 29: 1-9.
3. Partis L, Croan D, Guo Z, Clark, Coldham T, Murby J. Evaluation of a DNA fingerprinting method for determining the species origin of meats. ***Meat Science*** 2000; 54: 369-376.

4. Wolf C, Rentsch J, Heubner P. PCR-RFLP analysis or mitochondrial DNA: a reliable method for species identification. ***J Agric Food Chem*** 1999; 47, 1350-1355.
5. Stratil A, Peelman LJ, Van Poucke M, Cepica S. A HinfI PCR-RFLP at the porcine leptin (LEP) gene. ***Anim Genet*** 1997; 28: 371-372.
6. Janssen FW, Buntjer JB, Lenstra JA. Species identification in meat by using PCR-generated satellite-DNA probes. ***J Indust Microbiol Biotech*** 1998; 21: 115-120.
7. Winterø AK, Thomsen PD, Davies W. A comparison of DNA-hybridization, immunodiffusion, countercurrent immunoelectrophoresis and isoelectric focusing for detecting the admixture of pork to beef. ***Meat Science***1990; 27: 75-85.
8. Hunt D, Parkes HC, Davies ID. Identification of the species of origin of raw and cooked meat products using oligonucleotide probes. *Journal of Food Chemistry* 1997; 60: 437-442.
9. Montiel-Sosa JF, Ruiz-Pesini E, Montoya J, Roncales P, Lopez-Perez MJ, Perez-Martos A. Direct and highly species-specific detection of pork meat and fat in meat products by PCR amplification of mitochondrial DNA. ***J Agric Food Chem*** 2000; 48: 2829-2832.
10 Al-Rashood KA, Abdel-Moety EM, Rauf A, Abou-Shaaban RR, Al-Khamis KI. Triacylglycerols-Profiling By High Performance Liquid Chromatography: A Tool for Detection of Pork Fat (Lard) in Processed Foods J. ***J Liq Chromatogr*** 1995; 18: 2661-2672.