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Specific Polymerase Chain Reaction (PCR) Analysis of Raw Meats and Fats of Pigs for Halal Authentication

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Abstract: Species-specific polymerase chain reaction (PCR) analysis of a conserved region in the mitochondrial (mt) 12S ribosomal RNA (rRNA) gene was developed for species identification from raw pork and lard samples. Genomic DNA of pork and lard were successfully extracted and were found to be of good quality. The extracted genomic DNA was then subjected to PCR amplification targeting the specific regions of the 12S rRNA gene and produced clear PCR products on the amplification of 12S rRNA gene of 387 base pairs (bp) from pig species. The species-specific PCR identification yielded excellent results for identification of pig. This made it ideal for quality control purposes and a potentially reliable technique to avoid species adulteration for Halal authentication and verification.

Key words: Species identification · Pork · Lard · Species-specific PCR · 12S rRNA

INTRODUCTION

The identification of species origin forfood products is animportant issue to detect adulteration or fraudulent substitution. It is also as a protection for the consumers from the presence of unknown, less desirable species origin for economic, religious such as Islam and Judaism and public health reasons [1]. Furthermore, expensive and luxurious meat in the food market has been targeted for substitution or adulteration. Previously, the adulteration of meat products by the addition of low-cost meats from different species has been reported [2-4] and this obviously shows that this practice serves economic purposes. Hence, analytical methods for species identification are necessary in order to protect consumers from fraud.

Species identification methods based on the protein molecules such as isoelectric focusing (IEF) may not be successful in processed food products due to denaturation of proteins during food processing and often present cross-reaction with closely related species [5]. DNA techniques based on PCR have become very important and proposed as useful tools for the detection of several animal species [6, 7]. PCR easily amplifies target regions of template DNA in highly specific and sensitive manner. It is also characterized by a rapid processing time and low costs [1].

Species identification requires genetic sequences with greater variation. The mt-DNA genes examined to date appear suited for this level of resolution. These non-nuclear targets possess several advantages over nuclear genes: Each cell has thousands of identical copies hence tests targeted to mt-DNA provide a significant improvement in sensitivity and robustness over the single copy nuclear genes, they have a relatively high mutation rate than nuclear DNA making it easier to resolve differences between closely related species and they tends to be inherited through the maternal germline whereas the resulting lack of heterozygosity in the alleles under study simplifies the analysis [8-10]. Therefore, the mt-DNA 12S rRNA was selected in this work as the target sequence for species identification.

The aim of this study was to optimize a specific PCR detection method for species identification of pork and lard samples using a fragment of the mt-DNA 12S rRNA gene.

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MATERIALS AND METHODS

Samples: The meat and fat samples used in this study were derived from sheep, cow, chicken and pig. Sheep, cow and chicken samples were utilized as the controls. The samples were purchased from a wet market in Sri Kembangan, Petaling Street and PasarBorong Selangor, Malaysia. In order to prevent the enzymatic degradation of DNA, the samples were stored at -20°C prior to DNA extraction.

DNA Extraction: The meat and fat samples were subjected to DNA extraction according to the DNeasy® Protocol for animal tissue provided with the DNeasy® Tissue Kit (Qiagen, Hilden, Germany). Approximately 25 mg of meat and fat samples were blended using a blender (Braun AG, Frankfurt, Germany) and incubated with 180 µl ATL buffer and 20 µl Proteinase K at 55°C in a water bath to disperse the sample overnight until the tissue was completely lysed. Four µl RNase A (100 mg/ml) was added the following day and incubated for 2 min at room temperature. The sample was mixed by vortexing for 15 s. Two hundred µl AL buffer was then added to the sample, mixed thoroughly by vortexing before incubated at 70°C for 10 min. Two hundred µl ethanol (96-100%) was added to the mixture and mixed by vortexing to yield a homogenous solution. The homogenous solution was pipetted into the DNeasy® mini column sitting in a 2 ml collection tube and subjected to a centrifugation of 12,000 *g* for 1 min. The DNA bound to the column was washed in two centrifugation steps using 500 µl AW1 buffer and AW2 buffer to improve the purity of the eluted DNA. The purified DNA bound to the column was then eluted with 150 µl AE buffer and stored at 4°C until further application.

Oligonucleotide Primers: A pair of primer was employed in PCR reaction. The PCR primers used were 12SFW (5'-CCA CCT AGA GGA GCC TGT TCT ATA AT -3') and 12SR (5'- GTT ACG ACT TGT CTC TTC GTG CA -3'), which were published by Rodríguez *et al.*[11].

Optimization of the 12S rRNA gene PCR Amplification: Amplification reactions were performed in 25 µl volume containing 1x PCR reaction buffer (50 mMKCl, 10 mMTris-HCl, pH 8.3), 25 mM $MgCl_2$, 10 mMdNTPs, 10 pmol primer, 5 units/µl of *Taq* DNA polymerase (Finnzymes, Espoo, Finland) and 30 ng of extracted DNA which was measured by a spectrophotometer (A 260 nm) (Beckman Coulter, California, USA). Amplification were carried out in the gradient thermal cycler (MJ Research PTC-200 Peltier Thermal Cycler) under the following program: pre-denaturation of 93°C for 2 min, followed by 35 cycles of denaturation at 93°C for 30 s, annealing at 63 to 72°C for 30 s and extension at 72°C for 45 s. The synthesis was completed at 72°C for 5 min. The PCR products were electrophoresed through 2% agarose gel (Promega, Madison, USA) for about one hour at constant voltage (74 V) in 1x TAE buffer, pH 8.0 and stained by ethidium bromide. A 100 bp DNA ladder (Promega, Madison, USA) was used as DNA size marker. The gel photo was taken using the Syngene gel documentation system.

PCR Amplification of the 12S rRNA gene: The mt 12S rRNA gene was amplified in a total volume of 25 µl containing 30 ng of extracted DNA which was measured by a spectrophotometer (A 260 nm) (Beckman Coulter, California, USA), 1x PCR reaction buffer (50 mMKCl, 10 mMTris-HCl, pH 8.3), 25 mM $MgCl_2$, 10 mMdNTPs, 10 pmol of each primer and 5 units/µl of *Taq* DNA polymerase (Finnzymes, Espoo, Finland). Amplification of mt 12S rRNA gene in the Perkin-Elmer (Gene Amp PCR system 2400) thermal cycler was performed according to the following PCR step-cycle program: pre-denaturation of 93°C for 2 min to completely denature the DNA template, followed by 35 cycles of denaturation at 93°C for 30 s, annealing at 68°C for 30 s and extension at 72°C for 45 s. Final extension at 72°C for 5 min followed the final cycle for complete synthesis of elongation DNA molecules. Electrophoresis of 10 µl PCR products was performed at constant voltage (74 V) on 2% agarose gel (Promega, Madison, USA) for about one hour in 1x TAE buffer, pH 8.0 and stained by ethidium bromide. A 100 bp DNA ladder (Promega, Madison, USA) was used as size reference. The gel photo was taken using the Syngene gel documentation system.

Purification and Sequencing of the PCR Products: PCR products were purified according to the protocol provided in the QIAquick PCR purification kit (QIAGEN). One hundred and twenty five µl PB buffer was added to 25 µl PCR sample and mixed by vortexing. The sample was then applied to the QIAquick column and centrifuged at 17,900 *g* for 60 s. A total of 750 µl PE buffer was added and centrifuged at 17,900 *g* for 60 s. The column was then placed in a new 1.5 ml microcentrifuge tube. Thirty µl EB buffer was added into the column to elute the DNA and the column was centrifuged at 17,900 *g* for 1 min. The purified PCR products, one sample per each positive sample were sent to First Base Laboratories Sdn. Bhd. for sequencing service by using ABI PRISM® 377 DNA Sequencer (GMI Inc., Minnesota, USA) which

automatically analyzes DNA molecules labeled with multiple fluorescent dye. PCR primers were used in the direct sequencing for the purified PCR products. The obtained sequenced was then blast into the gene bank (NCBI database) to check for identification.

RESULTS AND DISCUSSION

Identification of species origin based on DNA method has received particular attention in recent years. The species identification of animal origin represents an important subject in the field of modern food control and new analytical methodologies are necessary. Methods currently used for species identification include the development of conserved mitochondrial or nuclear DNA primers for PCR amplification followed by complementary techniques such as sequencing or PCR-RFLP [12]. In this work, a single-step PCR assay has been optimized for the species identification from pork and lard samples.

DNA extraction using the DNeasy® Tissue Kit (Qiagen) has allowed suitable amount of extracted DNA for 12S rRNA gene PCR amplification. The quality of the extracted DNA from 25 mg of meat and fat samples was examined by electrophoretic analysis through a 1.2% agarose gel (Promega) and a single band of high intensity were appeared in the lanes. The extraction therefore proved to be highly efficient and yielded good quality DNA.

In the optimization of the annealing temperature for the 12S rRNA gene PCR amplification, 68°C was chosen based on the results.A single band of high intensity was observed in the lane where the migration of the amplicon of 12S rRNA gene PCR amplification at 68°C occurred.

Agarose gel electrophoresis of the PCR amplified products (Fig.1) from the pig samples resolved a single band of 387 bp in the detection of 12S rRNA gene amplification. The amplification yielded product of 387 bp fragment is consistent with the results reported by Rodríguez *et al.* [11]. No other bands wereobserved in the amplification mentioned which indicated the specificity of the system.

Species-specific PCR amplification analysis has shown to be a suitable method in detection of pig derivatives. The method shown to be a suitable method to control food authenticity because a specific target sequence can be detected [13]. In addition, PCR is a highly sensitive method, which had been proven by various species specific amplification [11,14-17]. The primers used in this study were specific because only a single band of the expected size regardless of the concentration of template mt-DNA extracted from meats and fats was observed in the specific PCR amplification.

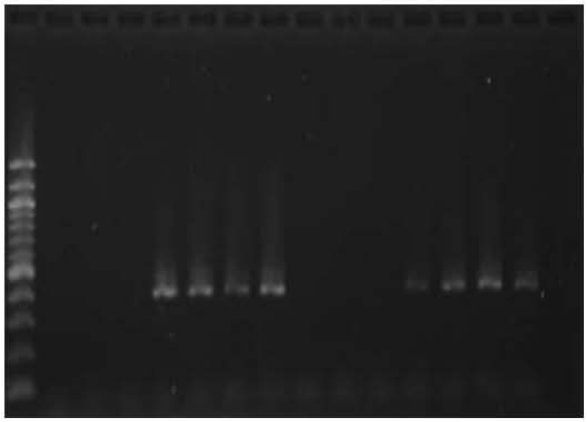

Fig. 1: Electrophoresis analysis of 12S rRNA PCR products amplified from meat and fat samples. M- 100 bp DNA ladder; 1- mutton; 2- beef; 3- chicken meat; 4,5,6 and 7- pork; 8- mutton fat; 9- cow fat; 10- chicken fat; 11,12,13 and 14- lard; N- negative control (no DNA).

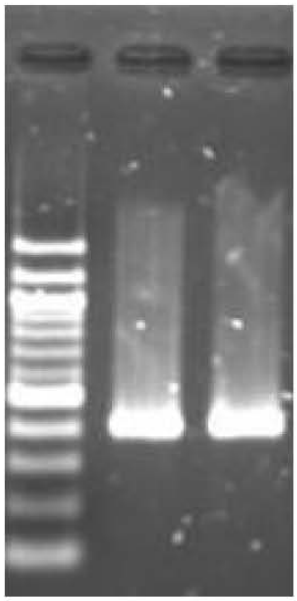

Fig. 2: Representative agarose electrophoresis gel photo of the purified PCR products from the positive samples (pig samples). M- 100 bp DNA ladder; DP1- pork; LP1- lard.

The PCR products were purified before they were sent for sequencing. Representative photo of agarose gel electrophoresis of the purified PCR products (Fig.2) shows a single band with high intensity at the expected size.

In the sequencing analysis of the 12S rRNA PCR amplification product, 100% and 99% identical were found when the obtained sequence from a representative pig's meat and fat samples compared with the database in

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gi|35757857|emb|AJ583551.1|Susscrofa partial 12S rRNA gene
 Length=404

 Score = 678 bits (342), Expect = 0.0
 Identities = 342/342 (100%), Gaps = 0/342 (0%)
 Strand=Plus/Minus
Query 1 AATATAGGTTATTTTTATTACTACATGCTTGAGGAGGGTGACGGGCGGTGTGTGCGTGCT 60
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct350 AATATAGGTTATTTTTATTACTACATGCTTGAGGAGGGTGACGGGCGGTGTGTGCGTGCT 291
Query 61 TCATGGCCTTATTCAATCAAGCACTCTATTCTTGATTTACTGCTAAATCCTCCTTTGGTT 120
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct290 TCATGGCCTTATTCAATCAAGCACTCTATTCTTGATTTACTGCTAAATCCTCCTTTGGTT 231
Query 121 TTTAGTTTCATAAAAACTTTCGTGTGGTGGATATTCTTATGTAGAAAATGTAGCCCATTT 180
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct230 TTTAGTTTCATAAAAACTTTCGTGTGGTGGATATTCTTATGTAGAAAATGTAGCCCATTT 171
Query 181 CTTTCCAACCCATAAGCTACACCTTGACCTAACGTTTTTATGTGCTATGATTGTGCTTAC 240
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct170 CTTTCCAACCCATAAGCTACACCTTGACCTAACGTTTTTATGTGCTATGATTGTGCTTAC 111
Query 241 TATTGTTCCTTTTTAGGGTTTGCTGAAGATGGCGGTATATAGGCTGAATTGGCAAGGGTT 300
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct110 TATTGTTCCTTTTTAGGGTTTGCTGAAGATGGCGGTATATAGGCTGAATTGGCAAGGGTT 51
Query 301 GGTAAGGTCTATCGGGGTTTATCGATTATAGAACAGGCTCCT 342
 ||||||||||||||||||||||||||||||||||||
Sbjct 50 GGTAAGGTCTATCGGGGTTTATCGATTATAGAACAGGCTCCT 9
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Fig. 3: Alignment of the representative obtained pig's meat sequence from the 12S rRNA PCR amplification with the sequence from the NCBI Blast Database.

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gi|35757857|emb|AJ583551.1|Susscrofa partial 12S rRNA gene
 Length=404

 Score = 670 bits (338), Expect = 0.0
 Identities = 343/345 (99%), Gaps = 0/345 (0%)
 Strand=Plus/Plus
Query 1 CCCGATAGACCTTACCAACCCTTGCCAATTCAGCCTATATACCGCCATCTTCAGCAAACC 60
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 35 CCCGATAGACCTTACCAACCCTTGCCAATTCAGCCTATATACCGCCATCTTCAGCAAACC 94
Query 61 CTAAAAAGGAACAATAGTAAGCACAATCATAGCACATAAAAACGTTAGGTCAAGGTGTAG 120
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 95 CTAAAAAGGAACAATAGTAAGCACAATCATAGCACATAAAAACGTTAGGTCAAGGTGTAG 154
Query 121 CTTATGGGTTGGAAAGAAATGGGCTACATTTTCTACATAAGAATATCCACCACACGAAAG 180
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 155 CTTATGGGTTGGAAAGAAATGGGCTACATTTTCTACATAAGAATATCCACCACACGAAAG 214
Query 181 TTTTTATGAAACTAAAAACCAAAGGAGGATTTAGCAGTAAATCAAGAATAGAGTGCTTGA 240
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 215 TTTTTATGAAACTAAAAACCAAAGGAGGATTTAGCAGTAAATCAAGAATAGAGTGCTTGA 274
Query 241 TTGAATAAGGCCATGAAGCACGCACACACCGCCCGTCACCCTCCTCAAGCATGTAGTAAT 300
 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 275 TTGAATAAGGCCATGAAGCACGCACACACCGCCCGTCACCCTCCTCAAGCATGTAGTAAT 334
Query 301 AAAAATAACCTATATTCAATTACACAACCATGCACGAAGANACAA 345
 |||||||||||||||||||||||| ||||| ||||
Sbjct 335 AAAAATAACCTATATTCAATTACACAACCATGCAAGAAGAGACAA 379
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Fig. 4: Alignment of the representative obtained pig's fat sequence from the 12S rRNA PCR amplification with the sequence from the NCBI Blast Database.

the NCBI Blast Database respectively (Figs.3 and 4). Sequencing of particular PCR products gave a completely reliable conclusion concerning species [18].

In conclusion, the extraction methods demonstrated in this study using DNeasy® Tissue Kit (Qiagen) provide a suitable routine extraction of genomic DNA from meat and fat samples. The extracted DNA was found to be suitable as PCR templates. The specific PCR method described herein is a useful and direct approach for the species- specific identification. The technique offers high sensitivity and specificity. Therefore, in this study the PCR technique is suggested to be a useful and powerful tool in species identification of pig's meat and fat in the aid of halal authentication.

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