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ANALYSIS OF PORCINE DNA IN SEVERAL FOOD PRODUCTS

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ABSTRACT

Species identification in food products has grown interest in recent years since these foodstuffs are liable targets for fraudulent labelling. Consumers of Halal products typically buying those with a Halal logo stamped on the packaging without doubt for its authenticity. DNA-based methods on PCR amplification were used to identify the presence of porcine DNA in the processed food samples. Genomic DNA of porcine was extracted using DNA-Sorb-C and subjected to PCR amplification targeting the mitochondrial 12S rDNA gene and porcine leptin gene (LEP). Out of 66 samples, 37 samples showed amplified product of 387 bp with mitochondrial 12S rDNA gene and 59 samples showed amplified products of 152 bp with LEP. The verification for Halal authentication of food products is significant for economical, religious and public health concerns.

Key words: food products, Halal, mitochondrial 12S rDNA gene, leptin gene, porcine, PCR

INTRODUCTION

The increase in Muslim population of approximately 1.6 billion Muslims worldwide has indicated the fast expansion of Islamic religion and these speculation has been agreed by Malaysia's Halal Industry Development Corporation (Nor Marini *et al.*, 2014). Concurrent to that issue, Halal will become worldwide symbol as quality assurance and people lifestyles (Hanzaee and Ramezani, 2011). Thus, the market opportunities and worldwide Halal market is profitable due to the increasing number of Muslim population in the world (Syed and Nazura, 2011). Furthermore, it generates bigger demand for Halal foods and products due to awareness among Muslims worldwide (Abdul Raufu and Ahmad Naqiyuddin, 2014). The demand for imported food products such as chocolates, biscuits and sweets are projected to escalate steadily over the next decade as a result of increasing consumption. Unfortunately, most of the imported food products does not have Halal Logo or doubted Halal Logo. As for Muslim and Jewish communities, the consumption of pork or pork related ingredients is strictly prohibited. Hence, Muslim community is very much concerned with food products and its contents. Currently, Malaysia is facing issues regarding the adulteration of chocolate products with porcine DNA. Adulteration usually includes substituting or diluting high value of raw materials with cheaper ingredients such as porcine or pork based ingredients (Kalivas *et al.*, 2014). The best methods to identify the presence of pork or porcine based byproducts in food products is by using molecular methods such as species specific PCR (Karabasanavar *et al.*, 2011), random amplified polymorphic DNA finger printing (Calvo *et al.*, 2001) and restriction fragment length polymorphism (RFLP) (Girish *et al.*, 2005). Through the development of DNA amplification by PCR, it is probable to detect the species in low amounts of DNA in the samples (Chi *et al.*, 2014). Nevertheless, species-specific PCR has superiority compared to other DNA-based methods in the matter of rapid and specific which is PCR-RFLP in particular require longer time, costly and requires specific analysis of results and Polymerase chain reaction-random amplified polymorphic DNA technique is very hard to repeat and requires strict amplification conditions (Karabasanavar *et al.*, 2014). Most of the forensic studies used the mitochondrial DNA because it contains a great copy number variation, maternal inheritance and great level of sequence variance (Rastogi *et al.*, 2007). Mitochondrial DNA is frequently used as genetic markers for species differentiation and the immanent numerous copies presence of mitochondrial genes creates an improved sensitivity of PCR analysis (Wang *et al.*, 2010). Mitochondrial DNA is the most extensively used to target genes for detection of animal (Fajardo

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et al., 2009). The species identification based on mitochondrial DNA is favored due to its varying specificity expressed in the species (Sahilah *et al.*, 2011). In addition, the leptin gene displays great homology of sequences in various species. (Stêpieñ- Poleszak *et al.*, 2009). In previous study, there was a successful identification of pork adulteration in food samples by the amplification of the 152 bp porcine leptin gene fragment (Ibrahim, 2008). The aim of this study was to determine of suspected Non Halal processed food products by using porcine mitochondrial 12S rDNA and porcine leptin gene.

MATERIALS AND METHODS

Raw materials

Raw materials used in this study were 66 suspected processed food products purchased from several hypermarkets around Shah Alam. There were eight types of chocolates, hard candies, soft candies, jellies, cheese, chocolate candy, biscuit and chewing gum. Pork sausage was used as positive control. The samples taken in segregated area which separated from Halal and Non Halal products. In addition, basket and counter for payment were also in segregated area which separated from Halal and Non Halal department.

DNA extraction from food samples

DNA was extracted by using DNA-sorb-C nucleic acid extraction kit (Amplisens, Russia) according to manufacturer's instruction. The sample was crushed with liquid nitrogen. An amount of 40 mg of sample was transferred into a 1.5 ml centrifuge tube. An amount of 400 µl of lysis reagent buffer and 17 µl of lysis reagent were added into the tube and vigorously mixed by pulsed vortexing for five seconds. Later, the sample was incubated at 64.0°C for one hour with occasionally stirring on vortex for five times for every ten to twelve minutes until the cells is completely lysed. After one hour, the tube was centrifuged at 12000 to 14000 rpm for five minutes. Then the supernatant was transferred using tips with aerosol filter into clean 1.5 ml centrifuge tube. The suspended particles and oil drops were not transferred. The tube was centrifuged at 5000 rpm for five seconds. After that, 25 µl of universal sorbent was added into the tube. Supernatant and universal sorbent were mixed well by vortexing the tube every two minutes. Then, the tube was centrifuged at 5000 rpm for one minute. The supernatant was removed using vacuum aspirator without aerosol barrier and also without disturbing the pellet. It was then followed by washing with 300 µl of washing Solution I and continued with stirring on vortex until sorbent was fully suspended. Then the tube was centrifuged at 5000 rpm for one minute. The supernatant was removed using vacuum aspirator without aerosol barrier and also without disturbing the pellet. After that, 500 µl of washing Solution II was added and continued mixing using vortex until sorbent was fully suspended. Then the tube was centrifuged at 10000 to 12000 rpm for one minute. The supernatant was removed using vacuum aspirator without aerosol barrier and without disturbing the pellet. The washing step using washing Solution II was repeated. Then, the supernatant was completely removed. Next, for sorbent drying, the tube with open cap was incubated at 64.0°C for five to ten minutes. In the last part of the protocol which is the elution step, 50 µl of TE-buffer was added. The tube was stirred on vortex. Later, the tube was incubated at 64.0°C for five to eight minutes and occasionally vortex while incubating for every minute. Then the sample was centrifuged at 12000 to 14000 rpm for one minute. The supernatant contains purified DNA and was ready for PCR amplification. Finally, all samples underwent each procedure accordingly similar to steps described above and all samples containing 50 µl of genomic DNA were stored at -20.0°C.

First PCR amplification

First PCR amplification was accomplished in 20 µl mixture containing 10 ng genomic DNA, 4 µl of 5x HOT FIREPol® Blend Master Mix, 0.2 µM of each forward and reverse primers and 4 µl of molecular grade water. In PCR amplification, the first set of primers used for specific PCR amplification were forward primer (5'-CCA CCT AGA GGA GCC TGT TCT ATA AT-3') and reverse primer (5'-GTT ACG ACT TGT CTC TTC GTG CA-3'). This application of primers was reported by Rodriguez and colleagues in 2003. This primer pairs were used to identify porcine mitochondrial DNA in food samples. As for the second batch of samples, similar PCR amplification procedures were carried out for second primers as reported by Neuenschwander and colleagues in 1996. The second set of primers were forward primer (5'-TGC AGT CTG TCT CCT CCA AA-3') and reverse primer (5'-CGA TAA TTG GAT CAC ATT TCT G-3'). This primer pairs were used to identify the pork leptin gene fragment in food samples.

Second PCR amplification

Second PCR amplification was accomplished by repeating the same procedure as in the first PCR amplification. Second PCR amplification results in 20 µl mixture containing 10 µl of DNA template of PCR product from the first PCR amplification, 4 µl

of 5x HOT FIREPol® Blend Master Mix, 0.2 µM of each forward and reverse primers and 4 µl of molecular grade water.

Gel electrophoresis of PCR products

Electrophoresis of the PCR product was performed using 2% (w/v) agarose gel prepared in 1× TBE buffer. Twenty µl of PCR product was loaded directly into separate wells on the gel. Then, electrophoresis was carried out at room temperature at a constant voltage of 100 volts until the blue dye migrates to two over third of the gel. When electrophoresis was completed, the comb was removed from the casting tray. The gel was visualized under Gel Documentation AlphaImager® (AlphaInnotech, California) for the detection and observation of the PCR products. The 100 bp DNA ladder (SolisBiodyne, Estonia) was used as marker.

DNA sequencing

An amount of 40 µl of PCR component was run for PCR amplification of DNA in thermal cycler (Bio-Rad, USA). The product of the PCR amplification was separated by electrophoresis on 2% (w/v) agarose gel to detect the presence of PCR band. An amount of 30 µl of each samples of processed food product, forward and reverse primer working solution at the 1:10 proportion was sent to Genomic Bioscience & Technology Company for DNA sequencing. Sequencing identity was confirmed using online program Clustal Omega and BLAST (Basic Alignment Search Tool) search for gene homology. Finally, all the sequences obtained were aligned for development of potential specific probes.

RESULTS AND DISCUSSION

In this study, DNA extractions from samples of processed food products including pork sausage as positive control were carried out. All procedures of DNA extraction were strictly performed according to the manufacturer's instructions in order to obtain high quality of DNA. The quality of the extracted DNA was examined by UV-VIS spectrophotometer analysis. A band of high intensity appeared from pork sausage sample (positive control). This indicates that the extracted DNA from pork sausage can be used as template for PCR amplification of the mitochondrial 12S rDNA gene and leptin gene.

Initially, there was no genomic DNA band detected for all processed food products samples tested. It is probably because of low yield of genomic DNA was extracted from all samples. In the second PCR amplification, it resulted with a clear band of target size for some of the samples. The amplification yielded product of 387 bp fragment which is in an agreement with the results obtained by Rodríguez and colleagues in 2003. This result indicates that the sausage and 37 samples of processed food products contained pork derivatives. In addition, PCR amplification of leptin gene yielded a band of 152 bp for the pork sausage and samples of processed food products which agreed with the results reported by Neuenschwander and colleagues in 1996.

In reference to Table 5.1, results indicated that sausage and 59 samples of processed food products contained pork derivatives and out of all, 31 samples were chocolate products. About 13 samples were detected with porcine mitochondrial DNA and 29 samples detected leptin gene. This gave an indication that there were porcine cells present in the chocolate products tested, where 10 samples were from hard candies product. Out of all, 6 samples were detected to contain porcine mitochondrial DNA and 6 samples detected on leptin gene. All soft candy product samples with presence of porcine derivatives were made up of fruit, chocolate and mint flavours. There were 5 samples which were jelly products containing porcine mitochondrial DNA and 6 samples were detected to contain porcine leptin gene. Gelatin was used in making the jelly products and it is believed that gelatin used in this jelly product consists of animal-based gelatin from pork derivatives. Only 1 sample used in this study was from cheese based products. This product also contained porcine mitochondrial DNA and porcine leptin gene. A total of 6 samples were chocolate candy. Only 2 chocolate candy samples contained porcine mitochondrial DNA but all six samples were detected to contain porcine leptin gene. A total of 4 biscuits samples of which 2 samples contained porcine mitochondrial DNA and only 3 samples were detected to contain porcine leptin gene. A total of 3 samples of chewing gum products contained porcine mitochondrial DNA and porcine leptin gene.

PCR products of tested food samples with positive presence of mitochondrial 12S rDNA gene and leptin gene were sent to Genomic Bioscience & Technology Company for DNA sequencing. Samples with mitochondrial 12S rDNA gene was further compared with BLAST to confirm that the sequences belongs to *Sus scrofa* mitochondrial DNA. BLAST search results showed a maximum identity of 99% for sample 60 and 91% for sample 40 and over query coverage of 100% with the available *Sus scrofa* mitochondrial DNA complete sequence in Genbank. Then, the sequences of the DNA were aligned in order to get a probe specific to Halal food. The probes were ACATAAAAACG TTAGGTCAAGGTG and CCCTAAAAAGGAA.

Table 5.1. Summary of Detection on Mitochondrial DNA and Leptin Gene for Eight Types of Processed Food Products

Types of Processed Food Products	Total	Detection on Mitochondrial DNA	Detection on Leptin Gene
Chocolates	31	13	29
Hard Candies	10	6	6
Soft Candies	6	5	6
Jellies	5	5	5
Cheese	1	1	1
Chocolate Candy	6	2	6
Biscuits	4	2	3
Chewing Gum	3	3	3

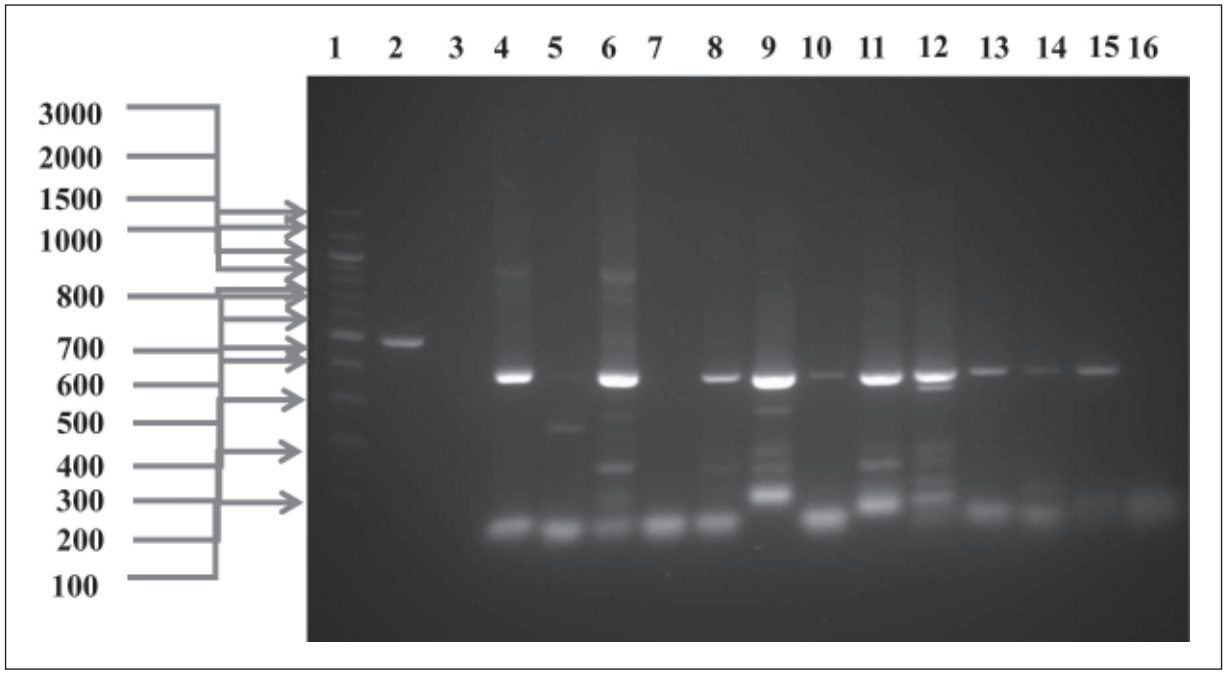

Fig. 1. Samples 40 to 51 of PCR Products for Mitochondrial 12S rDNA Gene Primer Pairs on 2% (w/v) Agarose Gel

Lane from extremely left:
Lane 1 : 100 bp DNA ladder
Lane 2 : Positive control
Lane 3 : Negative control
Lane 4 : Sample 40
Lane 5 : Sample 41
Lane 6 : Sample 42
Lane 7 : Sample 43
Lane 8 : Sample 44
Lane 9 : Sample 45
Lane 10 : Sample 46 A
Lane 11 : Sample 46 B
Lane 12 : Sample 47
Lane 13 : Sample 48
Lane 14 : Sample 49
Lane 15 : Sample 50
Lane 16 : Sample 51

CONCLUSIONS

In summary, PCR amplification of mitochondrial 12S rDNA gene yielded a band of 387 bp for the pork sausage and 37 samples of processed food products. These results indicate that sausage and 37 samples of processed food products contained porcine derivatives. In addition, PCR amplification of leptin gene yielded a band of 152 bp from the pork sausage and 44 samples from processed food products. Results obtained showed that sausage and 44 samples of processed food products also contained porcine derivatives. The food samples were concluded as Non Halal, even though the food samples were detected with only mitochondrial 12S rDNA gene or leptin gene. This was because

mitochondrial 12S rDNA gene and leptin gene was porcine specific. The alignment of the sequences was used to make up a probe specific to mitochondrial 12S rDNA gene. Two probes were obtained, one with 24 mers and 13 mers, respectively. The probes were ACATAAAAACGTTAGGTCAAGGTG and CCCTAAAAAGGAA.

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