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Halal analysis of raw materials, ingredients and finished bakery products using PCR and gene chip southern-hybridization for detection of porcine DNA

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Abstract

This study was conducted to investigate the sensitivity and detection of porcine DNA in raw materials, ingredients and finished bakery products by polymerase chain reaction (PCR) - southern hybridization on chip analysis. A total of 20 samples (n=20*3) with three replicates for each samples were obtained from a bakery factory located in Bangi, Selangor from January to December 2012. The sensitivity level of PCR-southern hybridization on chip was 0.001 ng. The species-specific oligonucleotide primers used in PCR-southern hybridization were targeted on the mitochondria DNA (mtDNA) of *cytochrome* b (cyt b) gene sequence, namely *cty* b biotin-labeled oligonucleotide primers. The amplicon from PCR amplification was 276 bp in size. None of the raw materials, ingredients and finished bakery product samples was positive towards porcine DNA, except for the positive control. The results in the present study demonstrated that the PCR- southern-hybridization technique on the gene chip (Olipro™ Porcine gene chip) is a sensitive tool for monitoring the porcine component in highly processed ingredients and finished bakery products.

Introduction

Bakery industries are one of the rapid growing on Halal food industries in Malaysia. The main Halal issues regarding the bakery products are their origin of their common ingredients used. Gelatin is one of the widely used raw materials in food and beverages, cosmetic and pharmaceutical due to its ability as foaming agent, gelling agent, plasticizer, emulsifier, stabilizer, thickener, moisture retention, improve texture and binding agent. Due to these characters, gelatin is commonly use in bakery and dairy products especially in yogurt, ice creams, cakes and cheese (Bhat and Karim, 2009).

Good productions of bakery products are possibly made with the addition of emulsifiers for improving the softness of the crumb, the tenderness and the fresh-keeping properties purposes (Funke *et al.*, 2009). Emulsifiers can be found naturally in fats and oils of animal like lard or vegetable origin like lecithin from soy beans. The concern of Halal issue regarding the emulsifier used in the bakery ingredient is the emulsifier that origin from lard. There are several cases reported regarding the adulteration of pig and its by-products by detection of porcine constituent in bakery products and ingredients that may due to the adulteration or fraudulent substitution. Addition of pig and its derived products as food ingredients in bakery products are possible due to low cost of materials such as gelatin and lard (Al-Jowder *et al.*, 1997).

The recovery of good quality genome DNA is possible from bakery products. Thus, the amplification of PCR using oligonucleotide primers to amplify the target gene is possible. PCR techniques using DNA amplification of specific target gene is a method of choice due to porcine DNA was detected based on mitochondria DNA (mtDNA). The mtDNA genome is preferable targeted compared to nuclear genome because it present in thousand copies per cell which in turn improve the possibility of detecting porcine DNA (Montiel-Sosa *et al.*, 2000). Thus, molecular DNA-based identification is possible to trace a small amount of porcine constituents in complex-composition and processed foods such as finished bakery products. The use of Olipro™ Porcine gene chip (PCR-southern hybridization) in detecting porcine DNA in raw materials, ingredients and finished bakery products is possible due to findings reported by Sahilah *et al.* (2012) in which porcine DNA could be detected in capsules which were highly processed. In this work, the sensitivity level and detection of porcine DNA were conducted in raw

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materials, ingredients and finished bakery products using polymerase chain reaction PCR-southern-hybridization gene chips.

Materials and Methods

DNA extraction

DNA extraction for pork meat was done using QIAGEN DNeasy Blood and Tissue Kit (Qiagen, USA) as provided by the manufacturer's instruction. A total of ~25 mg of raw meat was minced before the DNA were extracted using the kit. While, for the raw materials, ingredients and finished bakery products that were tested for Halal authenticity (wheat flour, pasteurized egg, unsalted butter, emulsifier, full cream milk powder, salt, sugar, baking improver, malt extract, baguette, wholegrain spelt mix, purple wholegrain mix, granbread mix, compressed yeast, cream cheese, FC sausage, chocolate chip cookies, chocolate paste, chocolate chips, butter flavour, T chocolate muffin and tomato strudel), the DNAs were extracted using Qiagen DNeasy Mericon Food kit (Qiagen, USA). A total of 0.2 g - 2 g of food sample was either grinded or homogenized before the DNA were extracted using that kit. The quantity and quality of purified DNA were determined by absorbance at 260 nm and 280 nm using spectrophotometer (MaestroNano, Maestrogen, USA).

PCR amplification

The biotin-labeled oligonucleotide primers were obtained from Olipro™ Porcine kit (Olipro Biotechnology Sdn. Bhd., Malaysia). The primers were designed for targeting the mitocondrial DNA of *cytochrome* b gene and positive results were indicated by single band of 276 bp. The internal control DNA was also supplied which produced a single band of 195 bp. Amplification was performed in a final volume of 50 µl as provided by the manufacturer. Each reaction mixture contained 50 µl volume containing 19.6 µl of master mix (biotin-labeled forward and reverse primers included), 0.5 µl of Taq DNA Polymerase, 24.9 µl of nuclease free water (NFW) and 5 µl of 50 ng DNA template. A negative-DNA control was performed by adding 5 µl of NFW and a positive control was performed by adding 5 µl of the known porcine DNA sample (~50 ng). The negative and positive controls were included in each PCR amplification in order to verify the PCR efficiency and to detect contamination. PCR was carried out in Eppendorf thermocycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 95°C for 5 min to complete denaturation of the DNA template, followed by 45 cycles of denaturation at 95°C for 30 sec, annealing for 30 sec at 55°C, extension at 72°C for 30 sec and final elongation at 72°C for 5 min. The amplification products were analyzed by electrophoresis using 2.5% agarose gel in 1X TAE buffer (40 mM Tris-OH, 20mM acetic acid and 1mM of EDTA; pH 7.6) at 90 V for 45 min and stained by ethidium bromide (EtBr). A 100 bp DNA ladder (BioLabs, England) was used as size reference. The gels were visualized using a gel documentation (AlphaImagerTM, USA). The result obtained was the biotinylated PCR products.

Southern-hybridization analysis on Gene Chip

Porcine specific nucleotide probes were fixed onto the gene chip membrane to capture specific targets generated from PCR reaction. The biotinylated amplicons were denatured at 95°C for 10 min and placed into ice immediately. The hybridization was carried out using Olipro™ Porcine gene chip and the amplicons were mixed with reagent A on the chips and incubated at 70°C in hybridization oven at maximum vibration for 1 hr. The addition of washing reagent B, C, D, E and F were done to the gene chip. The colorimetric development were done to the gene chip with reagent F, G and nitro blue tetrazoluene/ 5-Bromo-Chloro 3-indoyl-phospahate p-toluidine (NBT/BCIP) salt to give a color to hybridized probes. The chips were rinsed with reagent G and dried into oven at 37°C for 5 min. After hybridization, perfectly matched probe-target hybrid formed blue-purple precipitations on the chip as a result on enzymatic reaction. Chip pattern was read and identified by Olipro gene chip scanner with chip analysis software.

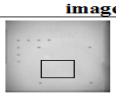
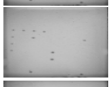
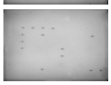
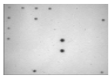
Interpretation of results

The positive detection of porcine DNA is shown by the grey color at two spots in the middle of the chip, while for the internal control, there appear eleven spots at the left and the upper part of the chip, including 4 spots located at every corner of the chips. However, no color will be formed in the middle of the chip if the result is negative. If the color does not appear at all internal spots, the results are not valid, thus the test should be repeated.

Sensitivity test

Seven mixed of wheat DNA (10 ng/µl) containing 0, 0.001 ng/µl, 0.01 ng/µl, 0.1 ng/µl, 1 ng/µl, 10 ng/µl of porcine DNA were prepared. PCR and southern-hybridization on gene chip were performed as mentioned above.

Table 1. Sensitivity test based on PCR-southern hybridization analysis using Olipro™ Porcine gene chip

Sample, concentration of porcine DNA combined with 10 ng/µl of wheat DNA (ng/µl)	PCR-southern hybridization analysis pattern using Olipro™ Porcine gene chip	
	Porcine DNA detection	Gene chip pattern image
0	-	
10	+	
1	+	
0.1	+	
0.01	+	
0.001	+	
0.0001	-	
Positive control	+	

grey color at two spots indicate positive for porcine DNA detection; no color at two spots indicate negative for porcine DNA detection

Results

Sensitivity test

PCR amplification products of all different concentration of porcine DNA combination with 10 ng/µl of wheat DNA by using Olipro biotin-labeled oligonucleotide primers of *cyt* b were shown in Figure 1. An internal control (IC) produced a band of 195 bp in size in all lanes including positive and negative control showed that all PCR reaction components were in good condition. The visibility of desired amplified DNA fragment (276 bp) was detected on the positive control, 10 ng, 1 ng and 0.1 ng of porcine DNA that were mixed with 10 ng of wheat DNA. No bands were observed in the lower concentration (0.01 ng, 0.001 ng and 0.0001 ng) of porcine DNA. However, by using further step that was gene chip hybridization, porcine DNA can be detected as low as 0.001 ng. Table 1 shows the sensitivity using PCR-southern-hybridization Olipro™ Porcine gene chip. The gene chip pattern image showed positive signals at 10 ng, 1 ng, 0.1 ng, 0.01 and 0.001 ng of porcine DNA that were mixed with 10 ng of wheat DNA. There was no colored signal at 0.0001 ng of porcine DNA mixed with 10 ng of wheat DNA.

Detection of porcine DNA in raw materials, ingredients and finished bakery products

The result of gene chip was interpreted by the pattern formed on the chip. The detection of porcine DNA in raw materials, ingredients and finished bakery products using PCR-southern hybridization Olipro™ porcine gene chip are shown in Table 2. Negative control showed no band was detected in the gel image and no colored signals were observed on the gene chip testing spot. The positive control showed band detection in the gel image and positive colored signals were observed on the gene chip testing spot. All 20 samples were shown to have no band detection in the gel image and no colored signals were observed on the gene chip testing spot except for the positive control.

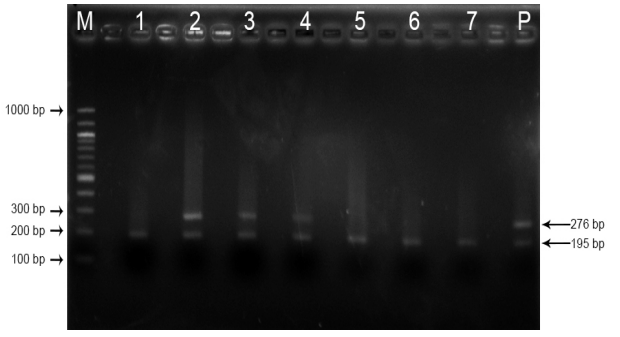


Figure 1. Sensitivity of the PCR method for porcine DNA detection using PCR OliproTM Porcine gene chip

Lane M: 100bp ladder. The amplification of 0 ng (lane 1), 10 ng (lane 2), 1 ng (lane 3), 0.1 ng (lane 4), 0.01 ng (lane 5), 0.001 ng (lane 6), 0.0001 ng (lane 7) of porcine DNA mixed with 10 ng of wheat DNA are shown. Lane P: Positive control.

The arrow showing 276 bp in length indicates the expected PCR product for positive porcine DNA while the arrow showing 195 bp in length indicates the PCR product for the internal control (IC).

Table 2. Porcine DNA detection based on PCR-southern hybridization analysis using Olipro™ Porcine gene chip on 20 samples of ingredients and finished bakery products

Sample*	Type	Porcine DNA detection
Nuclease free water	Negative control	-
Pork	Reference material	+
Canned food contain porcine ingredients	Positive Control	+
Wheat flour	Ingredient	-
Pasteurized egg	Ingredient	-
Unsalted butter	Ingredient	-
Emulsifier	Ingredient	-
Compressed yeast	Ingredient	-
Baking Improver	Ingredient	-
Malt extract	Ingredient	-
Baguette	Finished product	-
Cream cheese	Ingredient	-
Baking improver	Ingredient	-
Wholegrain spelt mix	Ingredient	-
Purple wholegrain mix	Ingredient	-
Granbread mix	Ingredient	-
Malt extract	Ingredient	-
Baguette	Finished product	-
FC sausage	Ingredient	
Chocolate chip cookies	Ingredient	-
Chocolate paste	Ingredient	-
Butter flavour	Ingredient	-
T chocolate muffin	Finished product	-
Tomato strudel	Finished product	-

*Each of the samples were tested in three replicates

Discussion

In the present study, the sensitivity test of PCR-southern hybridization on gene chip were evaluated before performing the detection of porcine DNA and its constituents on various ingredients and finished bakery products to justify whether that method is sensitive to verify the presence of porcine DNA in food samples. The used of PCR-southern hybridization on gene chip in identifying porcine DNA of soft and hard capsules in pharmaceutical products has been reported by Sahilah *et al.* (2012).

The sensitivity of PCR-southern hybridization was evaluated using porcine DNA mixed with wheat DNA. This technique was used and modified based on the previous study by Tanabe *et al.* (2007). Figure 1 shows the PCR amplification products of all different concentration of porcine DNA combination with 10 ng/µl of wheat DNA by using Olipro biotin-labeled oligonucleotide primers of *cyt* b. An internal control (IC) produced a band of 195 bp in size in all lanes including positive and negative control showing that all PCR were in a good condition. The visibility of desired amplified DNA fragment (276 bp) was detected on the positive controls, at 10 ng, 1 ng and 0.1 ng of porcine DNA that were mixed with 10 ng of wheat DNA. In the lower concentration (0.01 ng, 0.001 ng and 0.0001 ng) of porcine DNA, the band (276 bp) was not detected due to the invisibility on the agarose gel. However, it can be detected when it was hybridized onto nitrocellulose membrane on gene chip. Table 2 shows the sensitivity of the PCR-southern hybridization that yielded different results. The gene chip pattern image showed that the positive colored signals were observed on testing spot from 10 ng until 0.001 ng of porcine DNA that were mixed with 10 ng of wheat DNA. Thus, the sensitivity level of PCR-southern hybridization on the OliproTM Porcine gene chip was 0.001 ng of DNA per sample. The PCR-southern hybridization gene chip technique has shown to be more sensitive towards porcine DNA amplified gene due to its specific probes targeting *cyt* b on the gene chip (Sahilah *et al*., 2012). The gene chip showed the greatest level of sensitivity when very faint band in the agarose gel that cannot be seen by the naked eyes were visible on the gene chip.

Using the similar technique, 20 samples (n=20*3) with three replicates of various ingredients and finished bakery products were tested. As tabulated in Table 2 for the presence of porcine DNA, none of the samples were positive towards porcine DNA. Although the presence of porcine DNA was not detected, this technique is reliable and useful for highly processed product such as gelatin capsule (Sahilah *et al*., 2012). Various ingredients and finished bakery products undergone highly processed treatment such as heat and chemical, hence, there is a possibility to trace the presence of porcine DNA in such products. Thus, in the on-going study, bakery products treated with heat and spiked with different

percentage of porcine gelatin for further PCR-southern hybridization validation are undertaken.

In conclusion, although PCR method has shown to be simple and rapid, confirmation through southern-hybridization on the gene chip is recommended for Halal integrity and standard since the results on the gene chip shown very specific towards porcine DNA and demonstrated higher sensitivity of detection for up to 0.001 ng of porcine DNA. This present study has demonstrated that reliable and sensitive technology to detect porcine constituents in complex-composition and processed foods are necessary in Halal verification for Muslim consumers.

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