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Analysis of Porcine DNA in Gelatine Products using SYBR Green Real Time Polymerase Chain Reaction for Halal Verification

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Abstract

Real-time PCR was employed in this study to determine whether gelatine items from nearby marketplaces contained porcine DNA. The mitochondrial NADH dehydrogenase subunit 5 (MT-ND5) gene from pigs (Sus scrofa) was the subject of oligonucleotide primer design; the forward and the reverse primer sequences produced 161bp amplicon. SYBR Green fluorescent dye was used to detect swine DNA in samples of 22 different types of gelatine products, 12 of which had labels identifying their sources and indicating their certification as Halal, and the remaining 10 had neither labels nor certification information. The 40 cycles of positive amplication with a Ct value of 19.81 served as the criterion for determining the specificity of the primers. In this work, the detection threshold for porcine DNA is set at 0.001 µg/ml. The efficiency of the PCR in this study is 91.72%, and the R2 is 0.9985, which indicates good efficiency. The 12 labelled products exhibit positive amplifications for porcine gelatine products while exhibiting negative amplifications for both bovine and fish gelatine products in labelled samples. Nine out of the ten samples of gelatine products that were tested had positive amplifications without source or Halal labelling.

Keywords: *Porcine DNA; Gelatine; Real time PCR; Halal verification*

INTRODUCTION

Halal is a term derived from Arabic, which means legal or permitted (Asyraf et al., 2011). Bonne and Verbeke (2007) stated that for Halal, all food products will be allowed, except those that are clearly prohibited in the Al-Quran such as alcohol, pork, blood, meat from cadavers and meat from animals that are not slaughtered according to Islamic law. According to Kang et al. (2018), various food ingredients obtained from prohibited animal species, alcohol and harmful substances are also not allowed in the manufacture of Halal food products. Among these sources, the mixing of food with pork or its products in food products is prohibited. The absence of pig ingredients must be clearly verified on the commercial Halal food product label. Therefore, an accurate analytical method to assess the validity of Halal in food products is required. Gelatine (E441) is a type of protein that contains a high molecular weight and is used as a viscous agent in hard and soft capsules in the pharmaceutical field (Nikzad et al., 2017). Gelatine is also widely used in confectionery products (Shabani et al., 2015). According to GMIA (2012), gelatine can be made from various sources of collagen. The important sources of collagen for the manufacture of gelatine are

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cartilage, bone, tendon and skin of cattle and pigs. There is no plant-based gelatine source, and the relationship between gelatine and other ingredients to make gelatine from plants, such as from seaweed extract is non-existent. According to GME (2019), about 80 percent of the gelatine produced in Europe is made from pig skin. 15 percent is made from beef bone and 5 percent of gelatine is made from beef, pork and fish bones. However, products containing gelatine from pigskin are prohibited for use by Muslims and Jews.

METHODOLOGY

Samples

Pig, cow, and fish gelatine powder and sheets have been used. Positive control samples included powder and pig gelatine sheets, whereas negative control samples included cow and fish powders and gelatine sheets. Gelatine products with identified and unknown source were used. There are 12 labelled gelatine goods, while there are 10 non labelled gelatine products. For labelled products, there are five porcine gelatine products, five bovine gelatine products, and two fish gelatine products.

DNA Extraction

The QIAGEN DNeasy® Mericon TM Food Kit was used to extract DNA from raw meat, while the CONGEN SureFood® PREP Animal X Kit was utilised to extract DNA from gelatine powder, sheets, and food products. Using a BioDrop TM DUO UV / Vis Spectrophotometer (United Kingdom), the isolated DNA was evaluated for purity and amount.

Oligonucleotide Primers

The oligonucleotide primer of the pig (*Sus scrofa*) mitochondrial gene NADH dehydrogenase, subunit 5 (MT-ND5) was designed using Primer3Plus software (www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi). The specificity of the designed primers can be determined using the Basic Local Alignment Tool (BLAST) in the National Centre of Biotechnology Information (NCBI) GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) database. The oligonucleotide primers were synthesized by Apical Scientific (Selangor, Malaysia).

Amplification of DNA with Real-Time polymerase chain reaction (PCR)

The real-time SYBR Green polymerase chain reaction (PCR) was used in this study for the detection and quantification of pork DNA in gelatine products containing pork DNA. Real-time polymerase chain reaction (PCR) reaction system (ABI Stepone Plus) is used for the amplification of DNA samples. TOYOBO THUNDERBIRD® SYBR qPCR Master Mix is used as a reagent solution for this method. Compression was performed using 20 µL of solution for each tube containing 10 µL of

reagent TOYOBO THUNDERBIRD® SYBR qPCR Master Mix, 2 µL primers (1 µL for forward primer and 1 µL for reverse primer), 1 µL DNA template, and 7 µL ddH2O. Each sample in the reaction tube was labeled. No control template (NTC) containing distilled water was used to replace the DNA at each reaction performed on the sample (Kane and Hellberg, 2016). The real-time PCR cycles were as follows: pre-denaturation was 95°C for 60 seconds and denaturation was 40 cycles, 95°C for 15 seconds while annealing and extension were 60°C for 60 seconds.

Specificity of Real-time PCR

The primer specificity has been tested against three common species of animals including pig, cattle and fish. 10 µg/ml of the extracted DNA from those three species were analysed on real time PCR according to optimized condition for porcine specific primer. Different gelatine powders and sheets from pigs, cows and fish have also been used to test the primer specificity.

Real-time PCR Sensitivity and Efficiency

Real-time PCR sensitivity was done by performing tenfold serial dilution with pesticide DNA of 1 ng / µL, 0.1 ng / µL, 0.01 ng / µL, 0.001 ng / µL. The limit of detection have been determined by drawing a standard curve using Cq values with log values for the beginning of the DNA amount. According to Fajardo et al. (2008), real-time PCR efficiency can be calculated from the standard graphite slope with the formulation, E = [10 ^ ((- 1 / slope)) - 1].

FINDINGS AND DISCUSSION

Oligonucleotide Primers

In this study, oligonucleotide primers for the pig (*Sus scrofa*) mitochondrial NADH dehydrogenase subunit 5 (MT-ND5) gene were designed to be used in real-time polymerase chain reaction (PCR) using Primer3Plus software (www.bioinformatics.nl/cgibin /primer3plus/primer3plus.cgi). The forward primer sequence for pig is 5'-TAA TCC TCG TAA CCG CCA AC-3' while the reverse primer for pig is 5'-AAA TCC AAT GTC TCC GAT GC-3' for 161 bp amplicon size. Among the mitochondrial genes, NADH dehydrogenase subunit 5 has the appropriate target length; appropriate degree of intra-species conserved region and inter-species polymorphism; sequence databases for most animals and plants are available (Ali et al., 2015). PCR uses small DNA primers, about 20 to 30 base pairs that bind to the complementary region of the target nucleic acid sequence (Peaper and Landry, 2014). The length for both the forward primer and the reverse primer used is 20 bp. The suggested amplicon size for optimal amplification efficiency is between 50 to 150 bp (Dinoop et al., 2016). In this study, the designed primer amplicon size was 161 bp. Based on Singh and Pandey (2015), the primary GC content to obtain optimal efficiency for real-time PCR is 40% to 70% while the melting temperature, Tm is between 58°C to 60°C. The GC content can be calculated with the formula, GC%= (G+C)/(A+T+C+G) ×100% (Li and Du, 2014) while the melting temperature can be calculated with the formula, Tm = 4(G + C) + 2(A

+ T) (Borah, 2011). In this study, the GC content of the forward primer is 50% and the reverse primer is 45% while the melting temperature of the forward primer is 60°C and the reverse primer is 58°C.

Specificity of Real-Time PCR

Real-time PCR specificity analysis of forward primers and reverse primers specific for pig DNA only was carried out with DNA from raw pork, beef, chicken and fish meat as well as DNA from both pig, beef and fish gelatine powders and flakes. Primer specificity was confirmed with only positive amplification being obtained from pig DNA and both types of pig gelatine. DNA from other raw meats such as beef, chicken and fish as well as DNA from both types of gelatine other than pork, namely beef and fish, amplification for the DNA was not positive. During real-time PCR, there are at least three distinct phases for the curve. Initially, in the slow phase no amplicon was detected. Next, the exponential phase of the amplification and then the horizontal phase, the amount of the target in the original sample can be determined with precision when the number of cycles required by the signal to reach an arbitrary threshold (Tille, 2013). In real-time PCR, the threshold is determined significantly above the base line and typically in the middle region of the log-linear phase of the amplification signal. The number of cycles required for the fluorescence signal to cross the threshold is the quantization cycle (Cq), threshold cycle (Ct) or crossover point (Cp) (Bain et al., 2016). The Ct value is derived from the relative fluorescence and is related to the amount of starting material, the primary efficiency and the amount of fluorescence that exists for each cycle (Maddocks and Jenkins, 2016). A low Ct value indicates a high amount of target nucleic acid while a high Ct value indicates a low amount of target nucleic acid (Raharjo and Rohman, 2016). The results of real-time PCR specificity for this study are shown in Table 1.

Table 1: Specificity analysis for real time PCR

Sample Type	Label	Ct value	Tm (°C)
Raw Meat	Pig	19.81	81.43
	Cow	ND	ND
	Chicken	ND	ND
	Fish	ND	ND
Gelatine Powder	Pig S	29.54	81.88
	Cow S	ND	ND
	Fish S	ND	ND
Gelatine Sheet	Pig K	31.46	82.03
	Cow K	ND	ND
	Fish K	ND	ND

Negative Control	Nuclease Free Water	ND	ND

Sensitivity of real time PCR

Sensitivity analysis was performed with ten-fold serial dilutions of porcine DNA template. A total of four concentrations were used starting with 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL, 0.001 µg/mL. Pig DNA serial dilution concentrations and Ct values are as listed in Table 2.

Table 2: Pig DNA Serial Dilution Concentrations and Ct Values

DNA Concentration (µg/mL)	Ct value
1	20.16
0.1	23.29
0.01	27.07
0.001	30.70

For the concentration of pig DNA 1 µg/mL, the Ct value is 20.16; for 0.1 µg/mL, the Ct value is 23.29; for 0.01 µg/mL, the Ct value is 27.07; for 0.001 µg/mL, the Ct value is 30.70. This analysis has shown the limit of detection for real-time polymerase chain reaction to be 0.001 µg/mL. The detection limit or limit of detection (LOD) is the lowest DNA concentration in the sample for specific pig DNA amplification that can be detected using real-time polymerase chain reaction.

According to Wolf and Lüthy (2001), species-specific isolation with conventional PCR, the detection limit was found to be only 0.1 ng for pig DNA. The TaqMan real-time PCR technique is used for the detection and quantification of pork in meat mixtures and the detection limit is 0.01 ng/µl (Rodríguez et al., 2005).

Efficiency of Real-Time PCR

Analysis of the PCR efficiency value for pig DNA was calculated with the formula from the slope of the curved standard graph with the formula, E = [10^((-1/slope))-1] (Fajardo et al., 2008). Pig DNA (*Sus scrofa*) was used to carry out a ten-fold serial dilution, from 1 µg/mL to 0.001 µg/mL to obtain PCR efficiency values. After running serial dilutions, the real-time polymerase chain reaction method was performed. The Ct value of each sample was recorded and a standard curve graph was drawn. Figure 1 has shown the standard curve graph for PCR amplification of pig DNA with concentrations of 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL and 0.001 µg/mL.

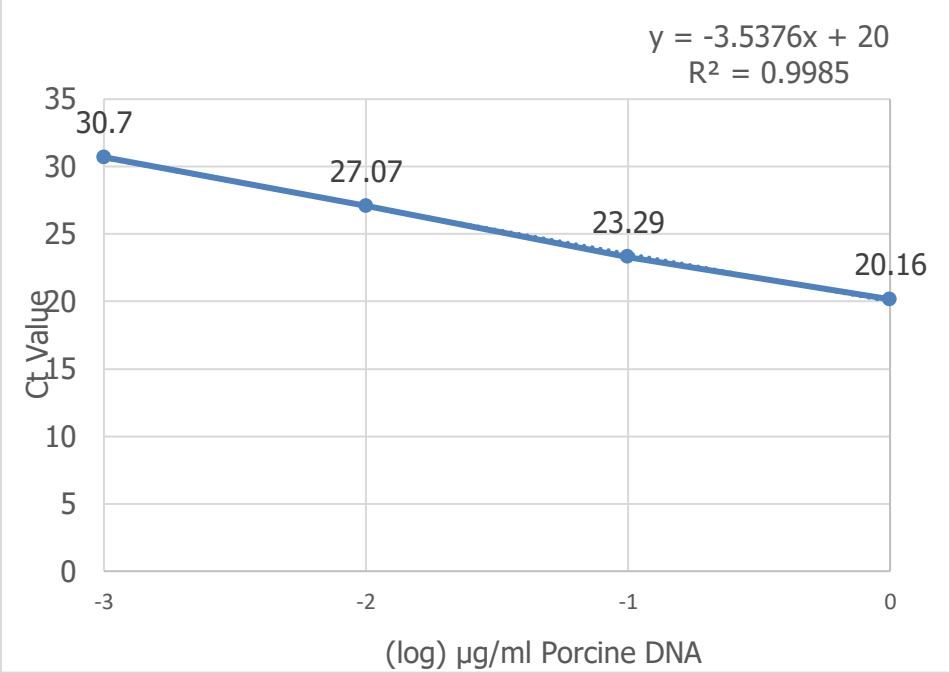

Figure 1: Linearity test, regression line and sensitivity parameters of the DNA amount (1, 0.1, 0.01 and 0.001) µg/ml from pig samples

The ideal graph linearity is r greater than 0.99 or R2 greater than 0.98, to ensure accurate quantification of the sample. The value indicates how linear the data is, if it gets a low value, this indicates variability in the dilution provided such as the amount of starter material that affects the amplification (Maddocks and Jenkins, 2016). The correlation coefficient, R2 for this study is 0.9985, has achieved the ideal linearity of the graph. This real-time chain reaction efficiency value is calculated from the slope of the standard curve graph with the formula, E = [10^((-1/slope))-1]. PCR efficiency should be in the range of 90% to 100%, −3.6 ≥ gradient value ≥ −3.3 (Marin et al., 2016). The slope of this graph is -3.5376, therefore, the efficiency value, E = [10^((-1/(-3.5376)))-1]=0.9172 or 91.72%. Therefore, real-time polymerase chain reaction for this study is efficient.

Detection of Labeled and Unlabeled Gelatine Products of Source and Legality with Real-Time PCR

Based on Table 3, DNA amplification occurs on gelatine products with source and halal labels for samples Pig 1, Pig 2, Pig 3, Pig 4 and Pig 5 while it occurs on gelatine products without source and halal labels for A, B, C, D , E, F, G, H and J. Amplification also occurred on positive controls, i.e. pig DNA from raw pig meat, pig gelatine powder and pig gelatine flakes. In addition, there was no amplification in the gelatine products with source and halal labels for Cow 1, Cow 2, Cow 3, Cow 4, Cow 5, Fish 1 and Fish 2 as well as in gelatine products without source and halal labels for sample I. Control negative, distilled water that has been used also shows no amplification.

Table 3 : Values of Ct, Tm and Presence of Pig DNA for Labeled and Unlabeled Gelatine Products Source and Halal

Sample Type	Labels	Ct Value	Tm Value	Results
Labelled Gelatine Products with known of origin	Pig 1	35.25 ± 0.870	82.47 ± 0.000	Positive
	Pig 2	32.24 ± 2.724	82.36 ± 0.075	Positive
	Pig 3	33.83 ± 1.995	82.40 ± 0.258	Positive
	Pig 4	30.19 ± 3.588	82.36 ± 0.224	Positive
	Pig 5	32.12 ± 4.861	82.25 ± 0.192	Positive
	Cow 1	ND	ND	Negative
	Cow 2	ND	ND	Negative
	Cow 3	ND	ND	Negative
	Cow 4	ND	ND	Negative
	Cow 5	ND	ND	Negative
	Fish 1	ND	ND	Negative
	Fish 2	ND	ND	Negative
Non labelled products with unknown of origin	A	34.53 ± 0.320	82.52 ± 0.087	Positive
	B	32.70 ± 1.415	82.37 ± 0.172	Positive
	C	24.20 ± 0.334	82.42 ± 0.086	Positive
	D	29.59 ± 2.926	82.32 ± 0.149	Positive
	E	28.38 ± 4.602	82.52 ± 0.087	Positive
	F	25.21 ± 4.730	82.13 ± 0.228	Positive
	G	31.75 ± 3.676	82.47 ± 0.149	Positive
	H	23.26 ± 2.478	82.22 ± 0.086	Positive
	I	ND	ND	Negative
	J	25.49±4.311	82.27±0.086	Positive

In this study, assay control is very important. Negative template control (NTC) is important to detect PCR contamination and unwanted amplification products such as primer dimers. A positive control and a negative control must be included for each real-time PCR test. With this, false

positive or false negative results can be avoided (Hyeon and Deng, 2017). In a previous study, pig DNA was found in ramen stock powder (Kang et al., 2018). According to Sepminarti et al. (2016), sweets containing gelatine were tested and found to have no pig DNA. According to Sudjadi et al. (2016), commercial capsules were tested and did not contain pig DNA.

CONCLUSION

A sensitive and efficient real-time PCR method using designed porcine species specific primers targeting the pig mitochondrial ND5 gene was used for the detection of pig DNA in labeled and non-labeled gelatine products. DNA amplification has been carried out successfully and results prove that this method is suitable for detecting pig DNA in gelatine products.

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