Rapid Detection of Porcine DNA by Real-Time Polymerase Chain Reaction (qPCR) on Imported Processed Foods

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ABSTRACT

A pair of porcine-specific primers defining a 145 bp region of the mitochondrial D-loop 443 were used to identify the presence of porcine DNA in ten samples of imported instant noodles and soft candies by Real-Time Quantitative Polymerase Chain Reaction (qPCR). This study was performed in three steps: sample preparation, DNA extraction, and DNA amplification using Real-Time PCR. The porcine DNA was amplified at an annealing temperature of 53^{o}C for 39 cycles. The result confirmed that three samples of instant noodles and three soft candies were found to contain porcine D.N.A. This method could effectively authenticate Halal products in processed foods, verify food labelling, and provide consumer protection.

Keywords*:* D-loop 443 Primer, Halal Authentication, Imported Food, Instant Noodle, Porcine DNA, qPCR, Soft Candy

INTRODUCTION

Indonesia is one of the countries with the largest Muslim community in the world, so halal certification becomes an obligation always afforded by either domestic food producers or food importers in Indonesia. It agrees with the regulations in Law Number 33 of 2014 regarding the guarantees for halal

products. The halal certificate is a written statement or fatwa issued by the Indonesian Ulema Council (MUI) regarding the halalness of food, beverage, drug, and cosmetic product produced by the company after going through a testing process by The Assessment Institute for Foods, Drugs, and Cosmetics, MUI (Hasan, 2014). Unfortunately, not all imported foods in the market have included a halal label on the packaging (Sakti et al., 2015).

One of the factors of non-halal food is the presence of pork in the composition, either the primary or additional ingredients (Hidayat et al., 2015). Some food products, especially those imported from non-Muslim countries, often use pork components in their production processes, for example, gelatine from pork bones as a thickener for soft candy (Hastuti & Sumpe, 2007) and pork fat as a flavouring in instant noodle seasoning (Jacinda, 2017). At the end of August 2021, the Central Bureau of Statistics of the Republic of Indonesia recorded that the total import value of candy products containing gelatine reached USD 14,933, and the instant noodles reached USD 11,865 (Statistic Indonesia, 2021). Therefore, a rapid detection method is needed to determine the presence or absence of pork within the food products imported to Indonesia so that the halal status can be immediately determined and spread in the market.

Various detection methods have been developed, such as Pork Detection Kit (PDK), which is based on antigen-antibody, yet the results are perceived as less accurate. Therefore, more specific and sensitive methods are needed, such as the Polymerase Chain Reaction (PCR) method, which is an enzymatic synthesis method for amplifying specific nucleotide sequences in vitro (Barbedo et al., 2016). Real-time-PCR has a higher sensitivity and specificity than the conventional one in detecting very few DNA samples. Real-time PCR analyzes quantitatively and does not require electrophoresis to confirm the results. The amplified DNA can be observed directly in the graph that appears as a result of the fluorescence accumulation from the reporter. The working principle of real-time PCR is detecting and quantifying based on the presence of a fluorescence reporter (Arya et al., 2005). The reporter used is SYBR Green, which will bind to double-strand DNA, and the fluorescence signal increases as the PCR product increases in the reaction (Fraga et al., 2014). The real-time PCR method also requires a primer. The type of primer is D-loop 443 because it can grow faster than other regions. The number of genes in mitochondrial DNA (mtDNA) reaches up to thousands of copies per cell and is suitable for use in many types of target sequences (Arini et al., 2018).

The current study focuses on the efforts to quickly detect the porcine DNA fragments in several imported food products widely available around Malang City, East Java, Indonesia. A rapid detection method with Real-Time Polymerase Chain Reaction (qPCR) is applied to detect porcine DNA fragments

in several imported brands of instant noodles and soft candies found in the market. It is expected to provide important information to Muslim consumers about imported food in Malang city.

MATERIALS AND METHODS

Materials

This study employed imported instant noodles and soft candies that did not have a halal logo, sold in the supermarkets around Malang City, East Java, Indonesia. They were purchased from Lai-Lai Mart and UD Sukses Jaya with 10 Korean instant noodle brands and ten soft candy brands. The lysis buffer consisted of 1% SDS (Merck), 0.1 M EDTA pH 8 (Sigma-Aldrich), 0.1 M NaCl (Merck), 50 mM Tris H.C.L. pH 8 (Sigma-Aldrich), T.E. buffer, which consisted of 0.1 M EDTA (Sigma-Aldrich) and 0.1 M Tris HCl (Sigma-Aldrich), proteinase K (Geneaid), Phenol: chloroform (1:1), Chloroform (Merck), Ethanol absolute (Merck), Ethanol 70% (Merck), and distilled water. The DNA amplification used Bio-Rad SYBR Green Master Mix, ddH2O, and primer Mitochondrial D-loop 443 (Forward: ACT AAT CAG CCC ATG CTC AC; Reverse: TGA CTG TGT TAG GGC CTT TG). The SYBR Green Master Mix contained dNTP, buffer, and DNA polymerase enzyme.

DNA Isolation

Samples were subtilized, mixed, and weighed as much as 25 grams. Then, 75 ml of distilled water was added and heated until dissolved. 500 µl was then taken and transferred into a microtube. 20 µl of proteinase K and 750 l of lysis buffer were added to isolate the DNA Then, it was incubated in a water bath at 55 C for 16 hours. After the incubation, the sample and control were centrifuged at 13,000 g for 10 minutes. The supernatant was then transferred to a new microtube and added ethanol: chloroform (1:1) as much as the volume of the existing sample. Then, it was centrifuged at 13,000 g for 20 minutes. A three-phase layer was formulated, and the first layer of DNA was taken. Then, the obtained supernatant was transferred into a new microtube and chloroform was added as much as the existing sample volume. It was centrifuged at 13,000 g for 20 minutes. The supernatant was then transferred to a new microtube. The addition of Phenol: chloroform (1:1) was repeated three times so that the obtained DNA purity was high. The DNA samples and control obtained were added with absolute ethanol twice as much as the sample volume. Then, they were stored in the freezer at -4 C overnight and centrifuged at 13,000 g for 20 minutes. The pellet in the microtube was then added with 500 µl of 70% ethanol.

Then, it was centrifuged at 13,000 g for 10 minutes. The pellets were added with ethanol 70% of and continued drying at 60 C until the ethanol evaporated. Finally, the pellet was added with 50 µl of T.E. buffer.

DNA Concentration and Purity Test

The DNA concentration and purity were tested using a UV/Vis spectrophotometric nanodrop with a 260 nm – 280 nm wavelength.

DNA Amplification with Real-Time PCR

The real-time PCR amplification process adopted the following formulation:

Table 1. Real-Time PCR Formulation

Materials	Volume 1x PCR (µl)
SYBR Green Master Mix	5.0
Nuclease-free water (ddH_2O)	3.5
Forward primer 0.06 µM	0.5
Reverse primer 0.06 µM	0.5
DNA sample	0.5

Then, the result was quantified using the following formula:

$$Expression\ level\ = 2^{(-dCq)} = 2^{(\sigma control - \sigma sample)} \quad \ldots\ldots(1)$$

RESULTS AND DISCUSSION

DNA Concentration and Purity of Samples

DNA concentration obtained in this study is relatively high (Table 2), more than 50 ng/µl, so it has a sufficient quantity for PCR amplification. The concentration of the isolated DNA is influenced by the isolation speed and the composition of the lysis buffer. The isolation speed factor produces an effect because the supernatant must be taken one by one at the cell lysis and precipitation stages so that some samples have DNA deposition (Komalasari, 2009). The purity level of the DNA isolation of each sample ranges from 1.3 to 1.9 (Table 2). The results of DNA purity below 1.8 indicate that protein contamination is still there, such as in the instant noodle samples 1, 2, 3, 4, 5, and 7, as well as the candy samples S, W, T, and 35. It might happen due to the lack of added proteinase K, requiring more proteinase K to lyse the protein (Ningsih et al., 2017).

Table 2. DNA Concentration and Purity of Instant Noodles and Soft Candies

Sample	Concentration (ng/µl)	Purity (λ260/280)
Pork	71.8	1.9
Chicken	62.2	1.8
Beef	130.7	1.9
Lamb	179.0	1.4
Instant noodle1	170.8	1.4
Instant noodle 2	110.4	1.7
Instant noodle 3	196.5	1.6
Instant noodle 4	208.5	1.5
Instant noodle 5	278.3	1.3
Instant noodle 6	79.2	1.9
Instant noodle 7	147.9	1.4
Instant noodle 8	82.7	1.8
Instant noodle 9	55.7	1.9
Instant noodle 10	97.3	1.8
Candy H	115.4	1.8
Candy W	152.5	1.7
Candy K	102.3	1.9
Candy T	104.4	1.7
Candy S	208.0	1.4
Candy M	81.5	1.9
Candy 31	142.7	1.8
Candy 33	79.2	1.8
Candy 36	57.0	1.8
Candy 37	83.6	1.8

Another possibility that causes protein contamination is the lack of repetition when adding phenol-chloroform during DNA extraction (Marwayana, 2015). In this study, the steps of adding phenol-chloroform were repeated three times. The results of DNA purity above 2.0 indicate that there is still contamination from compounds with smaller molecular weights, such as RNA The ethanol residues can also cause impure DNA during the incomplete drying process (Fatchiyah et al., 2011). Before the real-time PCR, the DNA concentration was adjusted to 50 ng/µl, so the results were not affected by the amount of DNA and the level of expression obtained under the same conditions.

Optimization of Real-Time PCR Conditions for DNA Amplification

The DNA amplification of pork, chicken, beef, lamb, and imported instant noodles and soft candy samples adopts a D-loop 443 primer to determine the presence of porcine DNA. The PCR amplification is usually performed for 35-40 cycles (Muladno, 2010). In this study, 39 cycles were used to amplify the DNA sequence and use SYBR Green as a marker. Table 3 shows the conditions of Real-Time PCR for the DNA amplification of the samples.

Table 3. Real-Time PCR Condition

Stage	Temperature (°C)	Time (second)	Number of Cycles
Initial denaturation	95	30	1
Denaturation	95	10	39
Annealing and Extension	53	30	

Optimizing the annealing temperature of DNA samples was performed by calculating the melting temperature (Tm) of primer using the formula {(G+C)x4}+{(A+T)x2}. The annealing temperature is 5ºC below the real Tm value. One of the successes of PCR amplification is influenced by the annealing temperature in the primer attachment process to the exposed DNA template. If the annealing temperature is too high, it can cause amplification failure because the primer is not attached to the DNA template. On the other hand, if the temperature is too low, it may let the primer attach to the other side of the DNA template so that it becomes non-specific. The annealing and extension stages are performed simultaneously. During the extension stage, a new DNA strand lengthening process occurs from the 5' to the 3' end of the template, starting from the primer attached. More than 1,000 bp will take about 120 seconds; 1,000 bp takes 60 seconds, and 500 bp only takes 30 seconds (Fatchiyah et al., 2011). The amplification of DNA samples in this study only requires 145 bp, so 30 seconds was applied to perform the extension and annealing stage simultaneously.

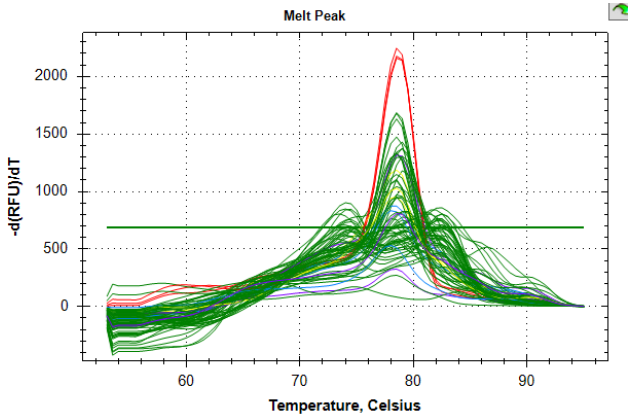

Figure 1. Melting curve of DNA Amplification

Real-Time PCR analysis makes it possible to observe during the reaction. It might happen because the presence of amplified DNA can be viewed on the graph due to the accumulation of fluorescence from the probe. The results of the real-time PCR program are processed and analysed using the CFX96 Maestro application and Microsoft Excel. Figure 1 shows only a single peak formulated on the melting curve. It indicates that the primer can amplify the PCR product specifically so that the signal obtained can be perceived as pure from the targeted gene, which is D-loop (Rasyid, 2015).

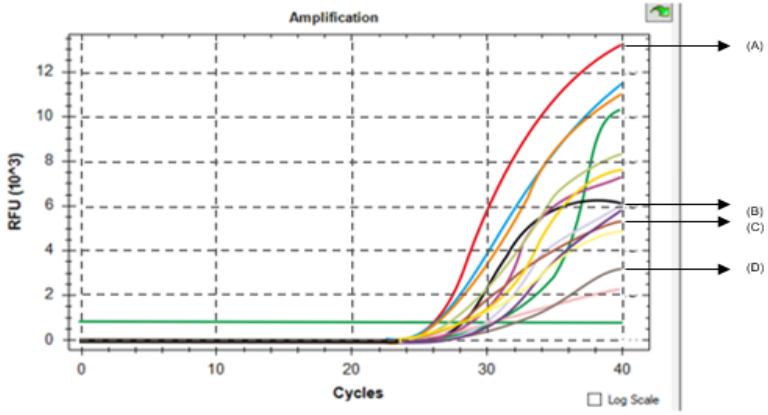

Figure 2. DNA Amplification Rate of Sample using CFX96 Maestro Application

Note :
A: Positive control (pork)
B: Negative control (lamb)

C: Negative control (chicken)
D: Negative control (beef)

In Figure 2, the x-axis indicates the number of cycles. In contrast, the y-axis indicates the RFU (10^3) or Relative Fluorescence Unit, namely the number of units in the fluorescence detection measurement. The green horizontal line is the threshold line indicating the minimum level of fluorescence detected in the sample and control DNA. The threshold line is obtained directly from the CFX96 Maestro application. The formation of the curve indicates the amplification rate of each sample and control. The Cq is the crossline between each sample's threshold and the amplification curve.

Sample Expression Level

The results of the gene quantification relatively use Real-Time PCR. There is a change in the relative expression level > 1 and < 1-fold. The relative expression level of 1-fold change indicates the expression of the D-loop primer in the sample is normal and proves the presence of porcine DNA in the sample. Therefore, the 1-fold change number is the minimum limit for the presence of the porcine gene in the sample. 1 (one) is the minimum agreed quantity for determining the target gene (Agilent, 2012).

Figure 3 shows that the positive control (pork) has an expression level of 1-fold change and refers to the housekeeping gene. Meanwhile, the negative controls (chicken, beef, and lamb) have an expression level of 0.54, 0.38, and 0.30. It indicates that the expression levels of the three negative controls are < 1 or below the housekeeping gene, so it is assumed that it does not contain porcine DNA Besides, the instant noodle samples numbers 2, 7, and 10 have an expression level of 1.08, 1.13, and 1.07, respectively, which indicates that they are > 1 or above the housekeeping gene. Thus, it is assumed to contain porcine DNA The instant noodle samples numbers 1, 3, 4, 5, 6, 8, and 9 have expression levels of < 1 or below the housekeeping gene. Thus, they are assumed not to contain porcine DNA.

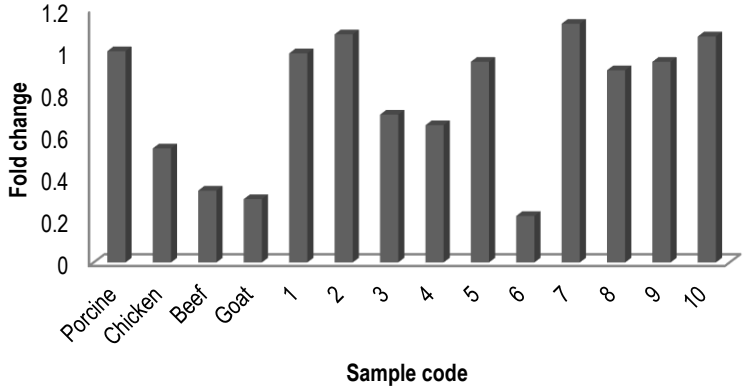

Figure 3. The expression level of 10 imported instant noodle samples

Figure 4 shows different levels of expression within the soft candy samples. Candy samples K, 31, and 33 show an expression level above 1-fold change, 1.12, 1.12, and 1.07, respectively. Therefore, they are assumed to contain porcine DNA. Meanwhile, the other samples (samples H, W, T, S, M, 36, and 37) show an expression level below 1-fold change, so they do not contain porcine DNA. The other sample has an expression level lower than 1, indicating that it does not contain porcine DNA.

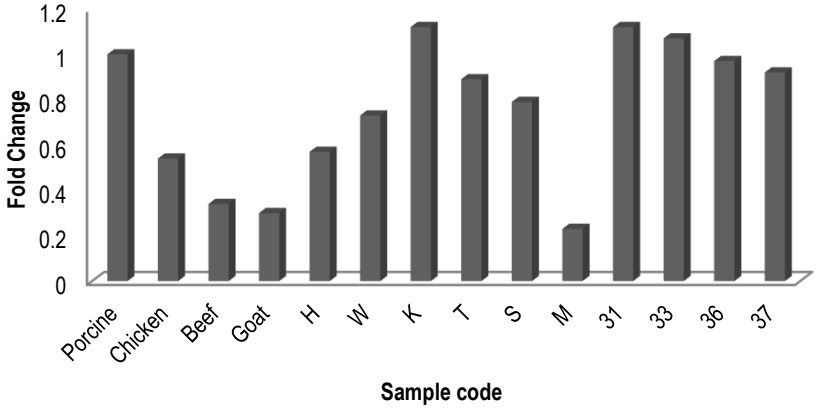

Figure 4. The expression level of 10 imported soft candy samples

CONCLUSION

The real-time PCR conditions were optimized for detecting porcine DNA: denaturation temperature of 95°C for 10 seconds, annealing and extension of 53°C for 30 seconds with 39 cycles. Under the test results of 10 instant noodle samples, there are three positive samples containing porcine DNA: samples 2, 7, and 10. Similarly, three soft candy samples contain porcine DNA: candy K, 31, and 33. This research concludes that non-halal imported food products are still available in the markets.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

AKW conceived the idea, planned the project, supervised the whole research, interpreted the data, reviewed, and edited the manuscript. R and A.S.R. performed the experiments and data analysis and was a contributor to writing the original manuscript. A.J.S. reviewed the manuscript. A.A.B. compiled the data and edited the manuscript.

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