Chapter 5

Molecular Based Method Using PCR Technology on Porcine Derivative Detection for Halal Authentication

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Additional information is available at the end of the chapter

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Abstract

Halal food and pharmaceuticals were taken into account as food and pharmaceutical products permitted to be consumed by Muslim according to Sharia law. Due to the development of science and technology, especially in food and pharmaceutical industry, some industries use non-halal components such as pig derivatives in food and pharmaceutical products to reduce the production cost. Non-halal components added in food and pharmaceutical products are difficult to detect visually due to close similarity between non-halal and halal components present in food and pharmaceutical products. Some of the methods already developed in the laboratory include spectroscopic methods using infrared radiation, Fourier-transform infrared spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy, chromatography-based methods, electronic nose, DNA-based and differential scanning calorimetric for pig component analysis. Food and pharmaceutical matrix is very complex to be analyzed; therefore, the signals obtained during chemical and biological analyses are very complex and are difficult to interpret. Even though the spectroscopy- and chromatography-based methods are able to determine the pig derivative component, there are some difficulties for the application in the field of blind samples, caused by the complex matrix of food or pharmaceutical. Among analytical methods, the polymerase chain reaction (PCR) based on the molecular genetic analysis was believed to be highly sensitive, valid and judgable as well as reliable for the analytical instrument. Therefore, this chapter describes some methodologies based on DNA technology such as conventional PCR using universal primer through restriction fragment length polymorphism or specific primer. This chapter also gives detailed information on the application of the real-time PCR using species-specific primer for porcine determination as well as for halal authentication.

Keywords: food, halal, molecular methods, pharmaceutical, pig derivative detection

1. Introduction

Today most of the Moslem countries are still growing in terms of life style, traveling, food and so on. Food is the primary need for humankind and cannot be separated from life; consequently, everywhere human beings live, including Moslems, they need food for living. In addition, awareness among Muslims on the need and necessity to consume only halal food is annually increasing, so the global market of halal food will be predominant in the future. It is understood that the production of halal food is not only beneficial to Muslims but also to food producers, since it will increase market space for Muslims especially and non-Muslims also who are interested in consuming halal food for health reasons. Human food production starts from the ingredients chosen, preparing the equipment, processing methods, packaging and labeling.

Human mobility which is increasing requires the availability of food according to the needs of each individual. Food security extends not only to safety and health issues but also to religious considerations. In the global realm, the need of tools to ensure that food is consumed safely and is a guarantee that it is free from non-halal materials requires a technology and non-halal detection application. This chapter is discussed in relation to some reliable methods of DNA-based analysis that is needed in justifying the presence of non-halal materials for Muslim consumers, in particular, the ingredients derived from pigs which are materials prohibited in Islam for consumption.

Identification of the presence of non-halal compounds becomes a very important problem in human food products, especially those containing prohibited substances such as ingredients derived from pigs. In the concept of food safety assurance, raw material data used is very important to be included. The world with a Muslim population of about 1.3 billion makes food security globally not only toward healthy and safe food products but also must show the halal aspect of the food product [1].

Authentication is the most important criteria and is always an issue in the field of food. The one that encourages the importance of authentication is the raw material, because it determines whether the food is deserved to be consumed. In Islam, "Halal" means that it is allowed or permitted and haram refers to anything that is not allowed and includes sin when violated. If used in relation with food and drink, halal means allowed to be eaten or drunk. It is mandatory for Muslims to eat halal food and consume halal products. For a Muslim, food should not only be healthy and of good quality, but more importantly, the food must be halal. Nowadays, many kinds of animal products have been commonly consumed and spread across countries without limitations. The relatively new products such as nuggets, sausage, burger, hot dog, meat ball and so on are widely accepted by consumers regardless of gender, ethnics and age. Further processing of animal products has given more economic advantage for the producers and convenience for the consumers.

In recent years, there has been an increasing trend in some countries for mixing prohibited substances, especially pigs in food products for the purpose of falsification to obtain economic benefits [2]. Identification becomes very important so that the status of halal food becomes clear. Two approaches that can be made to determine whether pig substances are present are analysis of the pig meat elements or pig material in food products.

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Various methods of detection of non-halal materials have developed rapidly. The method that has significantly developed is *FTIR spectrophotometry* [3] differential scanning calorimetry [4], liquid chromatography [5], immunoelectrophoresis [6], polymerase chain reaction (PCR)-RFLP DNA based methods [7] and real-time PCR [8].

Some of these methods are too laborious and time-consuming, consequently, an analytical technique offering rapid and reliable methods must be used. One of the promising methods suitable for routine analysis is polymerase chain reaction (PCR) [9]. The DNA-based analysis method has several advantages: DNA can be found in all cell types in an individual with identical genetic information. DNA is a stable molecule in the extraction process of several different types of samples.

Deoxyribonucleic acid (DNA) is a nucleic acid that contains genetic material and serves to regulate the biological development of all cellular life forms [10]. DNA contains genetic information stored in a nucleotide sequence and is used to synthesize all cell protein molecules and organisms. Another function is to provide information that is passed on to the cells or offspring of a child. Both of these functions require DNA molecules that serve as templates or models [11]. DNA has a double structure with components of pentose sugars (deoxyribose), phosphate groups and base pairs. DNA base pairs consist of purine and pyrimidine bases. The purine base consists of adenine (A) and guanine (G). The pyrimidine base is cytosine (C) and thymine (T) [12]. DNA is very important and widely used in analysis. DNA isolation is the key to successful identification of livestock derived from raw materials; therefore, the different methods of DNA isolation in each food stuff must be adapted to the conditions of the food type and food used in the diet.

2. DNA isolation stage

Identification of species based on DNA technology depends on the success of DNA isolation. The success of DNA isolation is the basis for continuing with the next test stage. Therefore, the isolation method should be known by any researcher who wishes to detect the species or foodstuffs. Work experience in the DNA isolation laboratory is a basic skill that needs to be known in order to isolate the various food matrixes. Some of the important factors that influence the success of DNA isolation are:

1. type of food
2. food matrix
3. cooking time
4. cooking temperature

DNA isolation has several stages, that is, (1) isolation of cells; (2) lysis of walls and cell membranes; (3) extraction in solution; (4) purification; and (5) precipitation. There are two principles in performing DNA isolation, that is, centrifugation and precipitation. The main principle of

centrifugation is to separate the substance based on molecular weight by providing a centrifugal force so that the heavier substances will be at the bottom, while the lighter substances will be located at the top. The centrifugation technique is carried out in a machine called centrifugation machine with varying speeds, for example, 2500 rpm (rotation per minute) or 3000 rpm [10].

Chemically, the destruction of cells is performed by utilizing chemical compounds such as ethylenediamine tetra acetic (EDTA) and sodium dodecyl sulfate (SDS). EDTA functions to destruct cells by binding magnesium ions (this ion serves to maintain cell integration and activity of nuclease enzyme which damages the nucleic acids). SDS is a kind of detergent that serves to destruct cell membranes. The proteinase K enzyme can be used to destruct proteins. Impurities due to cell lysis are separated by centrifugation. Then, the nucleotide molecules (DNA and RNA), which have been separated, are cleared of the residual proteins using phenol. In this process, a small part of RNA can also be cleaned. The RNAase enzyme is used to clean the residual proteins and polysaccharides from the solution. Purification of DNA can be done by mixing the DNA solution with NaCl, which serves to concentrate, separate DNA from the solution and precipitate DNA when mixed with ethanol [13].

The measurement of DNA concentration was done in two ways, that is, by spectrophotometer and by ethidium bromide fluorescence technique (EtBr). If the sample is pure, without large amounts of contamination such as protein, agarose, phenol or other nuclei, then the use of spectrophotometer that calculates the amount of UV irradiation absorbed by the bases is the proper way. The fluorescence technique of EtBr is used when the sample is contaminated [14].

DNA of ingredients in the form of fresh ingredients can generally be more easily isolated because most of the tissues have not been damaged. DNA of animal-derived food with cell structures easy to lysis is more easily isolated compared to a mixture of plant matrix ingredients which have stronger cell structures. Cooking duration will affect the quality and quantity of DNA that can be isolated, longer cooking duration will degrade the DNA; consequently, a lot of DNA fragments into smaller sizes, and as a result, at the time of amplification, there will occur difficulty with big-size amplicon target compared to small amplicon. The cooking temperature also has effect, the higher cooking temperature so the DNA in the food tends to be fragmented as a result for the size of the DNA with high fragment length target is difficult to amplify at PCR and difficult when identify the origin species of ingredients.

3. DNA isolation of animal tissue genome

The basic principle of total DNA isolation from the tissue is to break down and extract the tissue, so cell extracts composed of tissue cells, DNA and RNA cells are formed. Then, the cell extract was purified to produce a cell pellet containing total DNA [10].

DNA is usually isolated from animal tissue cells using methods that lyse cells but prevent DNA fragmentation. This step usually involves EDTA (ethylenediamine tetra acetic) which in the process will bind magnesium ions (the cofactors required by the DNase enzyme). Furthermore, the cell membrane is preferably solubilized with a detergent. If physical disruption is required, it should be done as minimum as possible. In this disruption process, the

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nuclease enzyme released from the cellular component can efficiently digest the nucleic acids, so the work of the nuclease enzyme must be inhibited. Cell disruption and most of the following steps should be performed at 4°C, using a glassware and an autoclave solution (the autoclave function is to destruct DNase activity in the apparatus or solution). After removing the nucleic acid from the cell, RNA can be removed by the addition of heat-treated RNase to inactivate the DNase of the contaminant (RNase is relatively stable to heat because of the presence of disulfide bonds which will cause the renaturation process when cooled).

Other major contaminants, that is, proteins, are eliminated with phenol solution or phenol-chloroform mixture (both will denature the proteins but do not denature nucleic acids). After the mixture is made into an emulsion, centrifugation is conducted. After centrifying, an organic part will be formed in the lower part and in the top aqueous layer, which is a layer composed of a denatured protein and inside it is contained DNA. The aqueous fluid is taken and deproteinized several times until no material is visible in the middle layer. Then, the DNA that does not contain protein is mixed with two parts of ethanol. DNA will be a precipitate, separate from the sample solution. After being centrifuged, the DNA pellets are then dissolved again [15].

4. DNA isolation of plant genome

Samples from plants are mostly found in food products both individually and food matrix such as sausage, meatballs and nuggets in which there are materials from animals and plants. The most commonly used method for plant DNA isolation is the *Cetyl Trimethyl Ammonium Bromide* (CTAB) method. CTAB is a common method used in plant DNA extraction because plant DNA contains polysaccharides and polyphenol compounds [16]. There are three major steps in the extraction of plant DNA, that is, the destruction of cell walls (lysis), separation of DNA from solid materials such as cellulose and proteins and DNA purification [17].

CTAB has the same function as SDS, which acts as a lipid solvent of the cell membrane. SDS is a kind of detergent that can emulsify lipids. After overnight incubation with ethanol absolute and ammonium nitrate, the DNA filaments are known and then centrifuged to obtain the DNA pellets. After the supernatant was removed, 70% of ethanol was added for next precipitation. Then, the pellet is dried and stored at −20°C [18].

5. DNA isolation of food products

Food products are a complex food mixture. Food products may contain PCR inhibitors such as polysaccharides, polyphenols and proteins [19]. In addition, food products have undergone several stages of processing, such as mechanical treatment, heating, chemical and enzymatic. As a result, DNA isolation from food products often has difficulties [20].

Some authors have compared methods of DNA extraction from food. Zimmerman et al. [21] compared nine different extraction methods of soybean; food samples showed that five of the methods had the extracted DNA damaged, while in four other methods (CTAB, Wizard, DNeasy

and nucleon Phytopure), the amount of DNA produced was relatively low but had good quality. Previously, Olexová et al. [22] have found good DNA extraction results using CTAB on soybean, corn and wheat products (flour, biscuits and instant porridge). Each sample in the food matrix needs the specific DNA isolation methods, as described by researcher [20] and they tried to compare four methods of DNA extraction (NucleoSpin kit, commercial and GeneSpin, CTAB, and Wizard method) applied to soybean processed products; CTAB method extraction showed the best results. Further, Sambrook [23] used CTAB modification by adding salt (NaCl) and twice as much CTAB volume in extracting food product samples for the identification of pork content.

6. DNA isolation in research practice

Today, the DNA isolation commonly uses DNA isolation kit as it is faster and is a simple method than the manual; consequently, the price is more expensive. The commercial kit has been available in varying amounts and benefits. However, the use of kits in DNA isolation is not suitable for student learning in college; therefore, the use of manual methods in student research is highly recommended. The use of kits in DNA isolation is usually simple and follows the procedures of the company, while manual DNA isolation requires the preparation of chemicals independently. The following table illustrates some basic methods and basic principles of DNA isolation (**Table 1**).

The results of DNA isolation research using the modified [14] method for sample of meatball and sausage as illustrated in **Figure 1** is as follows.

Food products are complex food mixtures which may contain inhibitors such as polysaccharides, polyphenols and proteins [19]. In addition, food products have undergone several

Method	The principle of DNA separation
TNES [24]	• Destruction of cell walls using EDTA, SDS and NaCl • Removal of protein with proteinase K • Removal of polysaccharide protein residues by using phenol, chloroform and isoamyl alcohol • Precipitation of DNA with saline solution (NaCl)
[14] modified	• Destruction of cell walls using EDTA and SDS • Removal of protein with proteinase K • Removal of polysaccharide protein residues by using phenol, chloroform and isoamyl alcohol • Precipitation of DNA with saline solution (NaCl)
CTAB	• Cell wall barrier and protein removal by extracting buffer (EDTA, Tris–HCl, NaCl, and K proteinase) • Precipitation of DNA with isoproponal

Table 1. The basic principle of DNA isolation in the DNA extraction method performed.

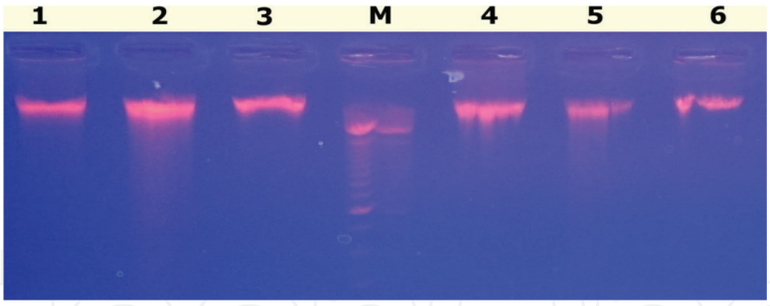

Figure 1. Result of DNA isolation of meatball and sausage of various species on 1% agarose gel: (1) 100% cow meatballs, (2) 100% chicken meatballs, (3) 100% pork meatballs, (M) 100 bp marker, (4) 100% cow sausage, (5) 100% chicken sausage, (6) 100% pork sausage.

stages of processing, such as mechanical treatment, heating, chemical and enzymatic treatments. As a result, DNA isolation from food products often has difficulties [20]. The complicities of sample affected the DNA quality [25] and the presence of additives on foods also affected the results of DNA isolation. Therefore, optimization and modification of the basic method to obtain DNA with good quality and quantity is required.

As this study applied the Sambrook method [14] without overnight incubation, the results showed that the quality of the DNA is not good enough and is marked by the appearance of DNA smear and some samples were not isolated (data not shown); therefore, the modification to prolong the lysis time and cell digestion by adding the incubation time to ±15 h (overnight) at a temperature of 55°C was made. The modification by adding the incubation time was intended so that the non-DNA molecules can be digested perfectly, thus minimizing the contaminants in the resulting DNA, since the sample was a processed meat consisting of a mixture of complex foods. The sample used in this study was a processed food product consisting of a mixture of meat (animal tissue) and non-meat (flour and spices) products that were considered as an inhibitor in the activity of DNA isolation.

The K proteinase was capable of digesting the cell completely after incubation of at least 10 h [23]. Meanwhile, previous research [26] founded that the duration of cell lysis depends on the shape of the extracted product. The more diverse the mixture and texture of the product, longer the time required. The optimization of ASL (Qiagien)-based extraction and purification of DNA by applying overnight to the cell lysis stage had been successfully performed for the identification of pork in processed meat products in Saudi Arabia [27]. The same method [28] was also used for *overnight* incubation at the lysis stage and digestion for the identification of pig DNA in food products. Chapela et al. [29] also used *overnight* incubation at the cell lysis stage in DNA extraction from canned tuna to identify species of animal origin.

DNA extraction of whole tissue is easier to do than processed meat such as meatballs, sausages and abon, where DNA has been degraded. The DNA obtained for analysis must be pure and intact to obtain good analysis.

The contaminants that are often present in the process of DNA extraction and purification are proteins [30]. DNA measurements were performed by comparing absorbance ratios at wavelengths of 260 and 280 nm (OD_{260}: OD_{280}) [15].

Repetition	Methods		
	Wasko [24]	Sambrook [14]	CTAB
1	1.64	1.91	1.70
2	1.65	1.91	1.70
3	1.63	1.91	1.70
Mean	1.64 ± 0.01	1.91 ± 0.00	1.70 ± 0.00

Table 2. Purity comparison of DNA isolation result of meatball sample with various methods [59].

The quality and purity of DNA is influenced by various factors including the isolation method and the type of food samples being prepared. The purity of DNA isolates from meatball samples with various methods of DNA isolation is described in **Table 2**.

Good DNA extraction methods not only provided good DNA results but also high DNA purity [30]. In the study, DNA purity measurements obtained from several DNA extraction methods had been performed. From **Table 2**, it was known that the purity of DNA obtained from the modified Sambrook method gave best results on meatball samples, (1.91 ± 0.00; 1.92 ± 0.00; and 1.87 ± 0.00). This showed that the method used could produce DNA with good purity. The value of DNA purity ranges from 1.8 to 2.0. The value of purity would be lower if there was contamination of protein or phenol [15]. Method of [23] obtained DNA purity value of 1.64 ± 0.01 (meatballs), while DNA purity value of the CTAB method was 1.7 ± 0.00 (meatballs). This value may indicate the presence of protein contamination in isolated DNA. Furthermore, in the practice of food, DNA isolation with a varying food matrix requires choice of method, method modification and development of the most suitable method according to food matrix condition whose DNA will be isolated.

7. Confirmation of pig species with conventional PCR

The wide variety of food products available on the market in the world seems favorable but leads to several fears for the Muslim community because the consumption of pork in food products is prohibited; therefore, some analytical methods offering fast and reliable results are continuously developed by some researchers. PCR-based methods have been applied through the PCR-RFLP method for the authentication of animal products especially meatballs in the market [9].

Polymerase chain reaction (PCR) is one of the most widely studied and widely used techniques of nucleic acid amplification in vitro. PCR is used to multiply the number of DNA molecules on a particular target by synthesizing new DNA molecules that complement the target DNA molecules through the aid of enzymes and oligonucleotides as primers in a thermocycle. The target length of DNA ranges from tenths to thousands of nucleotides whose positions are flanked by a pair of primers. The primer that is located before the target is called the forward primer and which is located after the target is called the reverse primer. The enzyme used as a novel printer of DNA molecules is known as the polymerase enzyme [31]. The length of the

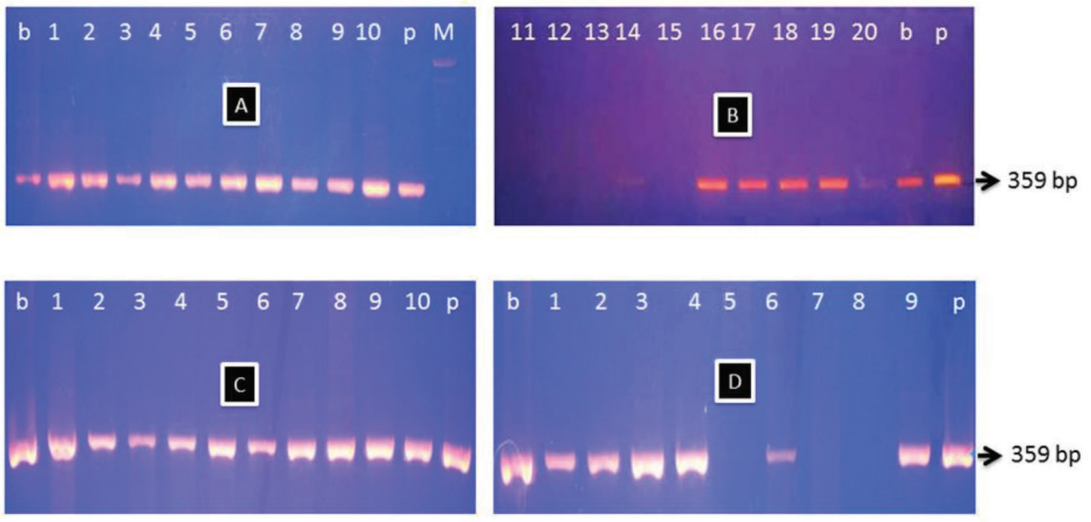

Figure 2. Amplification of mitochondrial cytochrome *b* DNA gene fragments 359 bp long in samples from meatballs products separated by 2% high-resolution agarose gel electrophoresis, M: Marker 100 bp DNA ladder (Invitrogen), A-D: PCR products of cytochrome *b* gene from 20 of commercial meatball samples from various regent in Indonesia b: Amplicon of beef DNA, and p: Amplicon product of raw pork DNA on [34]. Source: Aust-Asian J. Anim. Sci Vol. 27 No. 10.

DNA gene is 1.140 bp and has some stable sequences used for universal primers and several sequences of variables normally used for animal identification by the PCR-RFLP method [32].

A pair of primers was employed in PCR reaction (9), the PCR primers used were cytochrome b gen as follows: (5′-CCA TCC AAC ATC TCA GCA TGA TGA AA-3′) and CYTb2 (5′-GCC CCT CAG AAT GAT ATT TGT CCT CA-3′), as reported by Kocher et al. [33]. The PCR-RFLP was applied using mitochondrial cyt b gene in a final volume of 25 μl containing 250 ng of extracted DNA in order for the porcine identification with mega mix royal PCR buffer. Amplification was performed in PCR system 2400 (Perkin Elmer), programmed to perform the pre-denaturation step of 94°C for 2 min, followed by 35 cycles which is carried out with following steps: denaturation at 95°C for 36 s, annealing at 51°C for 73 s, and extension at 72°C for 84 s. Final extension at 72°C was conducted for 3 min for complete synthesis of elongated DNA molecules. The DNA amplicon then cleaved using BseDI restriction enzyme and results could able to differentiate porcine among other meat [9].

PCR has the potential sensitivity and specificity required to achieve detection of a target sequence from template DNA. Gene of cytochrome *b* was used for the amplification of PCR and resulted in DNA fragmentation of approximately 360 bp for bovine, chicken and porcine. These small amplicons are ideal for use with processed foods where DNA commonly was degraded. This result indicated that pig, beef and chicken DNA in processed meat was successfully amplified in PCR reaction (**Figure 2**). The result of PCR amplification was similar to that [35] which reported the presence of the 360 bp fragment. This result also indicated that the frying of meat products at 100°C for 15 min did not result in the degradation of DNA into small size (<200 bp). They studied the feasibility of using mitochondrial cyt b gene to detect porcine material in commercial food products from various markets and evaluated them for the presence of porcine DNA. Visualization through electrophoresis of PCR products clearly

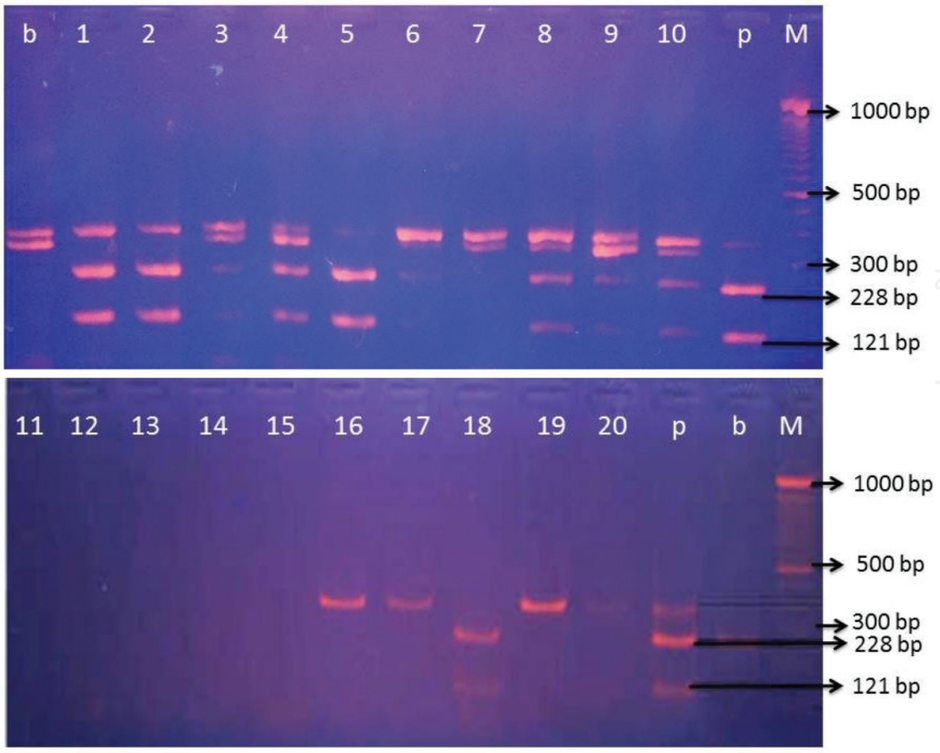

Figure 3. Restriction fragment length polymorphism using *BseD*I restriction enzymes. M: Marker 100 bp DNA ladder, lane 1–20: DNA fragment of different meatball samples from 20 commercial meatballs b: DNA fragment raw beef cytochrome *b* gene cleaved into different pattern, and p: DNA fragment of PCR product of raw pork cytochrome *b* gene cleaved into 228 and 121 bp [34]. Source: Aust-Asian J. Anim. Sci Vol. 27 no. 10.

detected porcine DNA, while no amplification occurred in others meat sources such as cattle, chicken, sheep and horse [36].

The result of the amplicon cleavage (**Figure 3**) showed that the enzyme of BseDI could differentiate pork meat among chicken, bovine or goat.

PCR product was digested using the *BseDI* restriction enzyme at 2 U/μl for 3-h incubation time. The result of analysis from various levels of pork in beef sausage and chicken nugget indicated that mixture of pork could be digested by the *BseDI* restriction enzyme and was detected until level 1%. *BseD*I cleavage bands visualized in the gel were enough and suitable for the discrimination of pork in processed food. *BseD*I endonuclease cleaved the cytochrome b gene products of pig species into two DNA fragments of 228 and 131 bp and did not cleave cytochrome b gene of beef and chicken [33].

Recent research [37] founded that pork DNA fragment of mitochondrial cytochrome b gen could be amplified well after cooking for 0–120 min (**Figure 4**), while cooking more than 120 min caused DNA degradation and failed to amplify mitochondrial DNA fragment, even for the PCR using more than 500 bp primer lengths. In addition, the amplification of 300 bp DNA fragment using specific primer showed DNA amplicon fragment in slight appearance. The band density decreased to less than 50% in sausage after cooking for more than 120 min. The 200 bp primer fragment could amplify all DNA samples from cooking for 120 min. Longer cooking time showed slighter appearance in gel electrophoresis and lower band intensity. Sausages were cooked for less than 120 min showed almost the same intensity, then decreased until about 60% band intensity. It

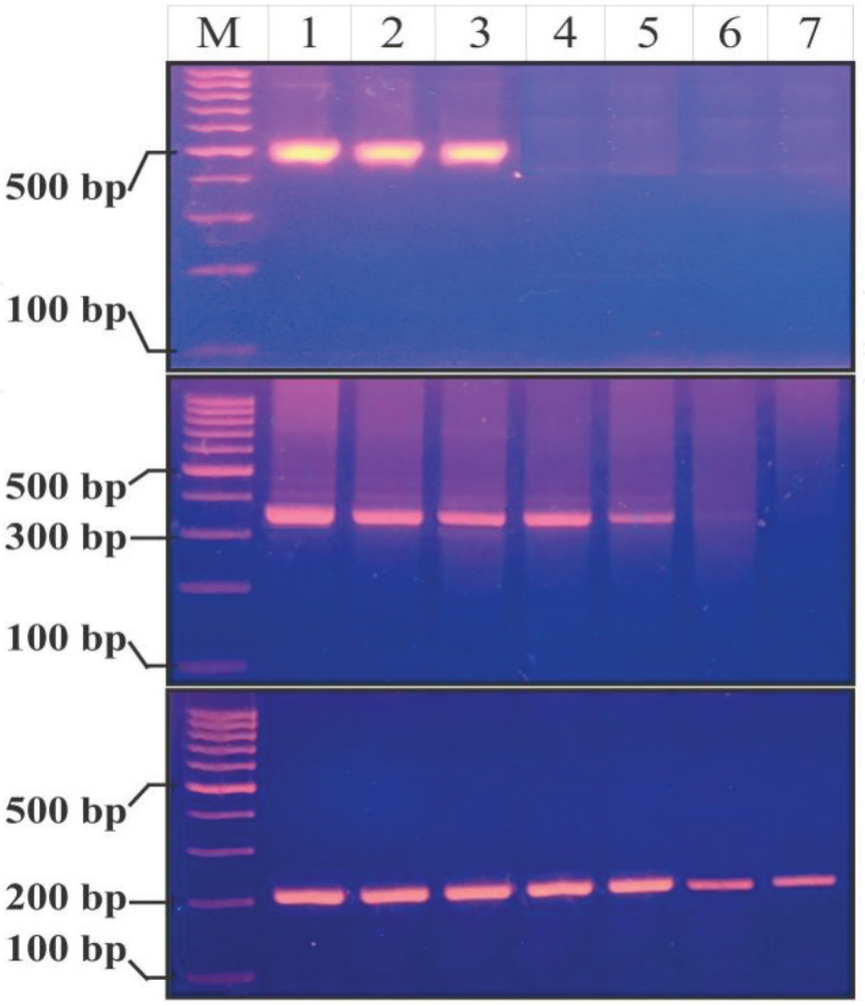

Figure 4. Amplification results of cooking time treatment. M: Marker, 1: no cooking (control), 2: 15 min, 3: 30 min, 4: 60 min, 5: 120 min, 6: 240 min, 7: 480 min. Above: 500 bp primer, middle: 300 bp primer, bottom: 200 bp primer.

indicated that the DNA from pork sausages after cooking for 120 min did not cause denaturation process, and the molecular weight did not decrease. The charged molecules can be separated in agarose gel electrophoresis according to smaller molecules migrating faster than larger molecules.

While cooking for more than 120 min, damage was caused on DNA fragment and could be amplified using species specific primer only above 200-bp length fragment (**Figure 4**). The DNA fragmentation occurred when meat was processed and cooked. The other researches [38] stated that the damaging of the DNA, as well as other meat structural changes (shrinkage of meat fibers, the aggregation and gel formation of myofibril and sarcoplasmic proteins, shrinkage and solubilization of the connective tissue, etc.), is due to the denaturation during heating.

8. Confirmation of pig species using real-time PCR

Compared to conventional end-point PCR, the main advantage of a real-time method is the possibility of performing quantitative measurements. The meat species identification using conventional PCR assays require the visualization using gel electrophoresis. The mistake analysis could increase with the chance of the cross-contamination or sample shifting; in addition, the process

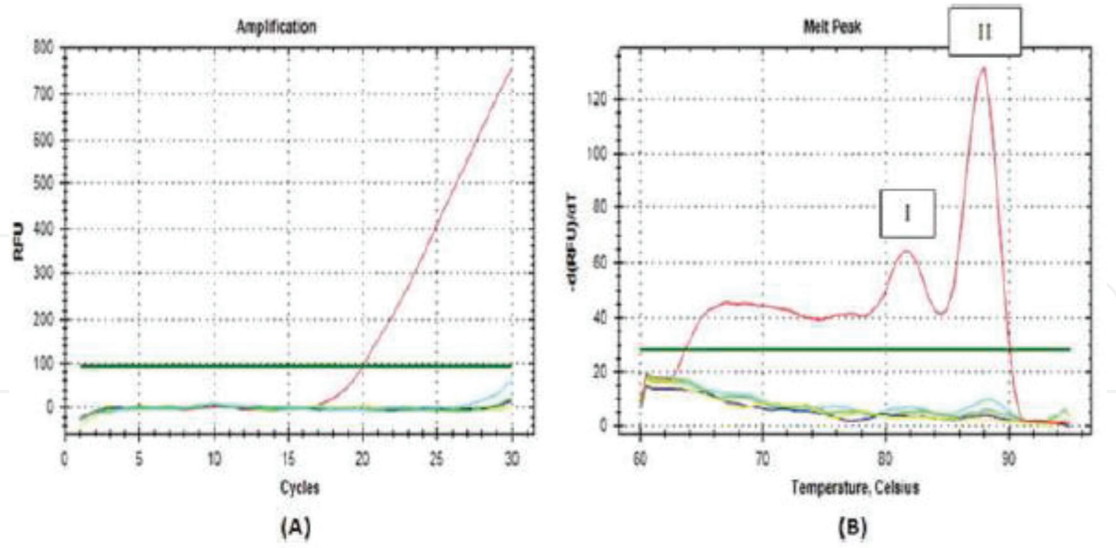

Figure 5. Amplification curves A: and melting peak; B: specificity of pork mitochondrial D-Loop686 primer on DNA from various raw meat. Red line: pork; yellow line: beef; dark blue line: chicken; green lines: goat; blue line: horse [42].

could not be automated. This condition may be a consequence because the PCR tubes must be opened after amplification. An alternative, shorter time-consuming analysis to differentiate the PCR products of various meat species would improve the assay. Melting curve analysis with an intercalating dye using a real-time PCR machine could remove the gel electrophoresis step, offered that the PCR products of the different species melt at different temperatures [39]. Basic principle of real-time PCR is the development of conventional PCR methods, in which amplification products can be monitored directly during each amplification cycle and can be measured. Real-time PCR testing allows for the identification of the early stages of the PCR process, which is more accurate than the endpoint analysis associated with agarose gel electrophoresis or polyacrylamide. The collecting data of real-time PCR was obtained using fluorescence molecules that showed a correlation between fluorescence intensity with PCR product abundance [40]. Real-time PCR is widely accepted as a powerful assay for the species identification and quantification of nucleic acid molecules. This procedure has higher sensitivity and specificity, larger dynamic range of detection, and less carry-over contamination risk. In the quantitative real-time PCR (qPCR) technique, amplification of the target gene is monitored by an increased fluorescence signal which enables direct assessment of the results after the PCR application without additional detection steps [41].

The amplification process of the PCR product can be observed from the beginning of the reaction to completion as shown in **Figure 5**. The number of PCR cycles is seen on the X-axis and the fluorescence of the amplification reaction on the Y axis. The amplification plot shows two phases, that is, the exponential phase followed by the non-exponential plateau phase. During the exponential phase, the amount of PCR products is approximately twice that of each cycle. In the plateau phase the reaction slows down (28–40 cycles) [43].

Threshold (initial limit) real-time PCR is the signal level which reflects statistically significant increases in baseline signal. The threshold is adjusted to differentiate the relevant amplification signal among the background. Typically, the real-time PCR instrument software automatically

adjusts 10 times the standard deviation of the baseline fluorescence value [44]. Threshold cycle or commonly abbreviated as Ct is the number of cycles during the fluorescence signal, that is, the reaction cuts the threshold. The Ct value is used to calculate the number of initial DNA copies because the value of Ct is the inverse of the initial number of targets. If the number of templates decreases, then the number of cycles required for amplification will increase [45].

In the initial reaction, the fluorescence on the base level and the increase in fluorescence is not detected at 1–18 cycles, although the product accumulates exponentially (**Figure 5**) until finally the amplification products sufficient to emit fluorescence signal that can be detected. The number of cycles when amplification occurs is called early cycle (threshold cycle or Ct). If the Ct value is measured in exponential phase, as long as the reagent is not limited to, *real-time* PCR can accurately and reliably calculate the amount of DNA present in the reaction. There are several types of fluorescence based on chemicals used for the detection of *real-time* PCR, which can be classified into four types: probe hydrolysis as TaqMan®, probe hairpin as molecule Beacon molecule, probe hybridization labeled with fluorescence (FRET) and the DNA intercalation dye as SYBR® Green and EvaGreen® [46, 47].

Fluorescent compounds *SYBR Green* are asymmetric cyanine compounds that have two aromatic rings connected by metena bridges, and each ring contains a nitrogen atom, and one of which is positively charged [48]. When SYBR Green is in the solution containing double-stranded DNA (ds-DNA), then the SYBR Green can be bound to ds-DNA and will emit a fluorescent signal at a wavelength of 254 (excitation) and 497 (emission) nm [49]. Characterization of fluorescence level that is obtained by SYBR Green can be seen through the Ct value, that is, at the DNA fragment amplification cycle, which was first detected. SYBR Green is widely used for the purpose of amplification optimization using qPCR, because it is easier and cheaper than probe [50].

The previous research [42] performed the real-time polymerase chain reaction using mitochondrial displacement loop686 and cytochrome b (cytb) gene primers for the identification specific pork DNA among other four types of DNA species: beef, chicken, goat and horse. The annealing temperature was at 62°C; however, the mitochondrial D-Loop686 showed high specificity to porcine DNA detection, as indicated by no amplification results that appeared on other species (beef, chicken, horse and goat meat) (**Figure 5**). It was proven that this additional peak does not result from primer dimer, since no amplification product on sample without DNA template is present.

The implementation of d-Loop 686 DNA and cyt b gene primers was carried out in order to identify pork DNA on laboratory prepared "dendeng" (Indonesian traditional beef Jerky). The results indicated that mitochondrial d-Loop686 primer had high specificity on pork DNA target. Results of experiment indicated that the primer has ability to detect pork DNA until the concentration of pork was 0.5% in the beef dendeng product with the consistent curve profile. The cytb gene primers also had a similar ability with d-Loop686 primer to amplify pork DNA until the pork concentration was of 0.5% in beef "dendeng". The results showed that these two primers, d-Loop region and the cyt b gene, had the capability to identify pork adulteration on the mixed pork-beef "dendeng" product at all variations of tested concentrations [42].

Soares et al. [8] applied a real-time PCR approach based on SYBR Green dye for the quantitative detection of pork meat in processed meat products. For the development of the method

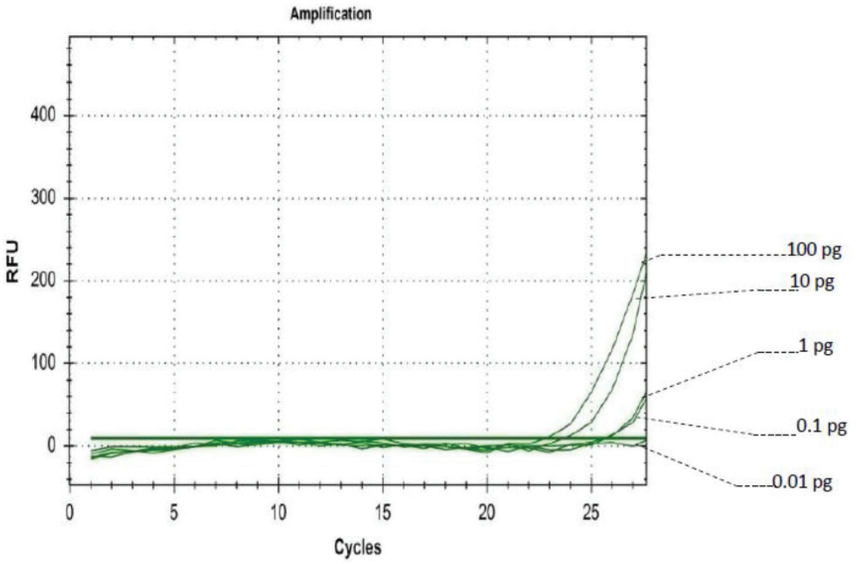

Figure 6. Amplification curves of binary reference mixtures (0.01–100 pg. of pork DNA) by real-time PCR with SYBR green I dye for pork detection and the detection limit was 0.1 pg.

analysis, binary meat mixtures containing known amounts of pork meat in poultry meat were used to obtain a normalized calibration model from 0.1 to 25% with high linear correlation and PCR efficiency. The method revealed high specificity by melting curve analysis and was successfully validated through its application to blind meat mixtures, which confirmed its adequacy for pork meat determination. The full applicability of the method was further demonstrated in commercial meat products, allowing verification of labeling compliance and identification of meat species in processed foods. **Figure 6** showed limit of detection of the real-time PCR which was able to detect the porcine DNA until 0.1 pg. contamination.

DNA degradation, inhibition or differences in the amount and quality of the DNA obtained from the food matrix sample was found. It meant that the processed meat products generally had several ingredients, including those from vegetable sources, and different processing treatments that might affect the target-gene amplification, the use of an endogenous control, which enables these variations to be controlled. Therefore, the structure of calibration curve based on real-time PCR normalization is essential for reliable quantitative analysis because this process controls variations in extraction yields and efficiency of amplification. The endogenous control allows verifying if amplification variations found with the species-specific primers were due to differences in target species content or to other factors such as (8).

Kesmen et al. [41] conducted the real-time PCR investigation and they concluded that TaqMan probe real-time PCR-based assay can be recommended for the detection of animal tissue by food control agencies or laboratories, and it might be a reliable and practical method for the determination of technically predictable contamination and/or intentional admixtures in complex processed meat products. **Table 3** described the application of the PCR-based technology on porcine determination of the commercial sample and **Table 4** showed the type of gene, fragment length and sequence of the primer already applied by some researchers.

Based on **Table 4**, the primer design for the application of the specific primer length target depends on the matrix sample. The sample with long period preparation and processing commonly

Species analysis	Issues	References
Animal Species	PCR assay for the identification of animal species in feedstuffs	[51]
Analysis of rat's meat DNA	Authentication of beef meatball from rat's meat	[52]
Analysis of pork DNA in dendeng	Authentication of "dendeng" from pork	[42]
Analysis of porcine gelatin	Analysis of porcine gelatin in capsule shell	[53]
Analysis of pork in meatball	Authentication of beef meatball from pork	[34]
Analysis of pork DNA	Identification of pig species in food product	[1]
Quantification of bovine, porcine, chicken and turkey species	Contamination and adulteration prohibited component in food and feed	[54]
Porcine Gelatin	Using gelatin in pharmacy and confectionery	[55]

Table 3. Analysis of porcine species-based PCR technology on commercial samples.

Genealogy	Specific primer sequence (5′–3′)	Products size (bp)	References
Mitochondrial Cyt b	5′ GCCTAAATCTCCCCTCAATGGTA 3′	212	[55, 56]
	5′ ATGAAAGAGGCAAATAGATTTTCG 3′		
12S rRNA-tRNA	5′ CTA AAT ATC AAG CAC CAT CAC A 3′	290	[51]
	5′ ACA TTG TGG GAT CTA CTT GG 3′		
cytochrome b	5′ CGC CTT ACG TTC TAA TGA CAT 3′	500	[9]
	5′ ATC CTA CTC T AG CC C ACC CA 3′		
ND5 gene	5′ CCATCCCAATTATAATATCCAACTC-3′	141	[57]
	5′ TGATTATTTCTTGGCCTGTGT GT 3′		
12S rRNA	5′ TGCAGTCTGTCTCCTCCAAA 3′	152	[58]
	5′ CGATAATTGGATCACATTTCTG 3′		
Mitochondrial Dloop 686	5′ GTTACGGGACATAACGTGCG –3′	114	[8]
	5′ GGCAAGGCGTTATAGGGTGT –3′		

Table 4. Various length of specific target DNA primers for porcine detection.

the DNA degraded into 100–200 bp, consequently if the PCR method applied using more than 300 bp DNA target. Some food matrix need long time preparation, conse.

9. Conclusion

It is common that food should consider an individual with health, culture, life style, religious faith, budget and choice. Food products without pork samples on the ingredients list, or even labeled 'pork-free', obtained from the commercial market and retail stores that could

be confirmed by PCR-based methods. Many experiments showed the detection limit of less than 0.1% porcine material level. This data showed that some samples could be considered contaminants of porcine materials. Reviews of these findings showed the value of such an analytical approach to guarantee the commercial products in world free trade. PCR-based method is proposed to be a reliable and sensitive protocol for routine analysis.

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References

[1] Erwanto Y, Abidin MZ, Sismindari X, Rohman A. Pig species identification in meatballs using polymerase chain reaction-restriction fragment length polymorphism for halal authentication. International Food Research Journal. 2012;**19**:901-906

[2] Aida AA, Che Man YB, Raha AR, Son R. Detection of pig derivatives in food products for halal authentication by polymerase chain reaction-restriction fragment length polymorphism. Journal of the Science of Food and Agriculture. 2007;**87**:569-572. DOI: 10.1002/jsfa.2699

[3] Rohman A, Sismindari X, Erwanto Y, Che Man YB. Analysis of pork adulteration in beef meatball using Fourier Transform Infrared (FTIR) spectroscopy. Journal of Meat Science. 2011;**88**:91-95. DOI: 10.1016/j.meatsci.2010.12.007

[4] Marikkar JMN, Lai OM, Ghozali HM, Che Man YB. Detection of lard and randomized lard as adulterants in refined-bleached-deodorized palm oil by differential scanning calorimetry. Journal of the American Oil Chemists' Society. 2001;**78**:1113-1119. DOI: 10.1007/s11746-001-0398-5

[5] Marikkar JMN, Ghazali HM, Che Man YB, Peiris TSG, Lai OM. Distinguishing lard from other animal fats in vegetable oils admixtures of some liquid chromatographic using the data coupled with multivariate analysis of data. Journal of Food Chemistry. 2005;**91**:5-14. DOI: 10.1016/jfoodchem.2004.01.080

[6] Necidová L, Renčová E, Svoboda I. Counter immunoelectrophoresis: a simple method for the detection of species-specific muscle proteins in heat-processed products. Veterinární Medicína – Czech. 2002;**47**(5):143-147. DOI: 10.17221/5818-VETMED

[7] Erwanto Y, Abidin MZ, Rohman A, Sismindari X. Using PCR-RFLP. BseDI enzyme for authentication in pork sausage and nugget products. Media Peternakan. 2011;**34**(1): 14-18. Bogor: EISSN

[8] Soares S, Amaral JS, MBPP O, Mafra I. A SYBR green real-time PCR assay to detect and quantify the pork meat in processed poultry meat products. Meat Science. 2013;**94**:115-120. DOI: 10.1016/j.meatsci.2012.12.012

[9] Erwanto Y, Yuliatmo Y, Sugiyono, Rohman A, Sismindari X. Species specific polymerase chain reaction (PCR) assay for identification of pig (*Sus domesticus*) skin in "Rambak" crackers. In: Proceedings of The 1st International Conference on Tropical Animal Science and Production, July 26-29, 2016; Bangkok

[10] Faatih M. Isolation and digestion chromosomal DNA isolation and digestion of chromosomal DNA. Research Journal of Science & Technology. 2009;**10**:61-67

[11] Martin DW. Biokimia (Harper's Review of Biochemistry). 20th ed. Jakarta: EGC; 1987

[12] Yuwono T. Biologi Molekular. Jakarta: Penerbit Erlangga; 2010

[13] Sulandari S, Zein MSA. Free Practice Laboratory DNA. Zoology Field. Bogor: Biology Research Center, Indonesian Institute of Sciences; 2003

[14] Sambrook J, Fritch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989. pp. 16-17

[15] Sambrook J, Russel DW. Molecular Cloning: A Laboratory Manual. 3rd ed. Vol. 1. New York: Cold Spring Harbor Laboratory Press; 2001

[16] Jose J, Usha R. Geminiviral. Extraction of DNA from a highly mucilaginous plant (*Abelmoschus esculentus*). Plant Molecular Biology Reporter. 2000;**18**:349-355

[17] Surzycki S. Basic Techniques in Molecular Biology. Berlin/Heidelberg/New York: Springer; 2000

[18] Tan SC, Yiap BC. DNA, RNA, and protein extraction: The past and the present. Journal of Biomedicine and Biotechnology. 2009;**2009**:10. DOI: 10.1155/2009/574398. Article ID 574398

[19] Terry P, Jain M, Miller AB, Howe GR, Rohan TE. Dietary intake of folic acid and colorectal cancer risk in a cohort of women. International Journal of Cancer. 2002;**97**:864-867

[20] Mafra I, Silva SA, EJMO M, CSF S, Beatriz M, Olivera PP. Comparative study of DNA extraction methods for soybean derived food products. Food Control. 2008;**19**:1183-1190. DOI: 10.1016/j.foodcont.2008.01.004

[21] Zimmerman A, Lüthy J, Pauli U. Quantitative and qualitative evaluation of nine in different methods for nucleic acids extraction on soya bean food samples. Zeitschrift fuer Forschung und Lebensmittel Untersuchyng A. 1998;**207**:81-90. DOI: 10.1007/s002170050299

[22] Olexová L, Dovičovičová Ľ, Kuchta T. Comparison of three types of methods for the isolation of DNA from fl ours, biscuits and instant paps. European Food Research and Technology. 2004;**218**:390-393. DOI: 10.1007/s00217040872

[23] Sambrook J. Mini-preparation (isolation and purification of plasmid). 2009. Available from: http://www.all-about-molecullar-wordpress.com. [Accessed: January 25, 2012]

[24] Wasko AP, Martins C, Oliveira C, Foresti F. Nondestructive genetic sampling in fish. An improved method for DNA extraction from fish fins and scales. Hereditas. 2003;**138**:161-165. DOI: 10.1034/j.1601-5223.2003.01503.x

[25] Nuraini H. Development of repetitive sequence element 1 (PRE-1) as a molecular marker for detecting material pig on processed meat products [dissertation]. Bogor: Graduate Program in Bogor Agricultural University; 2004

[26] Primasari A. Sensitivitas gen sitokrom B (cyt B) sebagai marka spesifik pada genus rattus dan mus untuk menjamin keamanan pangan produk asal daging [thesis]. Bogor: Institut Pertanian Bogor; 2011

[27] Alaraidh IA. Improved DNA extraction method for porcine contaminants, detection in imported meat to the Saudi market. Saudi Journal of Biological Sciences. 2008;**15**:225-229

[28] Che Man YB, Aida AA, Raha AR, Son R. Identification of pork derivatives in food products by species-specific polymerase chain reaction (PCR) for halal verification. Food Control. 2007;**18**:885-889. DOI: 10.1016/j.foodcont.2006.05.004

[29] Chapela MJ, Sotelo CG, Pérez-Martín RI, Pardo MA, Pérez-Villareal B, Gilardi P, Riese J. Comparison of DNA extraction methods from muscle of canned tuna for species identification. Food Control. 2007;**18**:1211-1215. DOI: 10.1016/j.foodcont.2006.07.016

[30] Tun-Nguyen CT, Son R, Raha AR, Lai OM, Clemente Micheal WVL. Comparison of DNA extraction efficiences using various methods for the detection of genetically modified organisms (GMOs). International Research Journal. 2009;**16**:21-30

[31] Muladno. Teknologi Rekayasa Genetika, Edisi Kedua. Bogor: IPB Press; 2010

[32] Minarovič T, Trakovická A, Rafayová A, Lieskovská Z. Animal species identification by PCR-RFLP of cytochrome b. Scientific Paper: Journal of Animal Science and Biotechnology. 2010;**43**:296-299

[33] Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Wilson AC. Dynamic of mitochondrial DNA evolution in animal: Amplifications and sequencing with conversed primers. Proceedings of the National Academy of science of the USA. 1989;**86**:6169-6200

[34] Erwanto Y, Abidin MZ, Muslim EYP, Sugiyono S, Rohman A. Identification of pork contamination in meatballs of indonesia local market using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Asian Australasian Journal of Animal Science. 2014;**27**:1487-1492

[35] Aida AA, Che Man YB, Wong CMVL, Raha AR, Son R. Analysis of raw meats and fats of pigs using polymerase chain reaction for halal authentication. Meat Science. 2005;**69**:47-52. DOI: 10.1016/j.meatsci.2004.06.020

[36] Tanabe S, Miyauchi E, Muneshie A, Mio K, Sato C, Sato M. PCR method of detecting the pork in foods for verifying allergen labeling and for identifying hidden pork ingredients in processed foods. Bioscience, Biotechnology, and Biochemistry. 2007;**71**:1663-1667. DOI: 10.1271/bbb.70075

[37] Erwanto Y, Yuliatmo R, Fitriyanto NA, Abidin MZ, Sugiyono S, Rohman A. DNA isolation and pig species detection on sausage with various cooking temperatures and times. In: Proceedings of the 2nd International Conference on Tropical Agriculture; October 26-27, 2017; Yogyakarta

[38] RWD R. Electron microscopy of bovine muscle: II—The effects of heat denaturation on post rigor sarcolemma and endomysium. Meat Science. Published by Faculty of Animal Science, Gadjah Mada University, Yogyakarta; 1989;**26**:281-294. DOI: 10.1016/0309-1740(89)90013-2

[39] Thanakiatkrai P, Kitpipit T. Meat species identification by two direct-triplex real time PCR assays using low resolution melting. Food Chemistry. 2017;**233**:144-150. DOI: 10.1016/j.foodchem.2017.04.090

[40] López-Andreo M, Lugo L, Garrido-Pertierra A, Prieto MI, Puyet A. Identification and quantitation of species in complex DNA mixtures by real-time polymerase chain reaction. Analytical Biochemstry. 2005;**339**:73-82. DOI: 10.1016/j.ab.2004.11.045

[41] Kesmen Z, Sahin F, Yetim H. PCR assay for the identification of animal species in cooked sausages. Meat Science. 2007;**77**:649-653. DOI: 10.1016/j.meatsci.2007.05.018

[42] Maryam S, Sismindari Raharjo TJ, Sudjadi RA. Determination of porcine laboratory contamination in jerky prepared using mitochondrial D-Loop686 and CYT b gene primers by real time polymerase chain reaction. International Journal of Food Properties. 2016;**19**:187-195. DOI: 10.1080/10942912.2015.1020434

[43] Real-Time PCR Applications Guide. Bio-Rad laboratories, Inc., USA [Internet]. 2006. Available from: http://www.gene-quantification.com/real-time-pcr-guide-bio-rad.pdf

[44] Anonymous. Basic of Real-Time PCR, Life Technologies Corporation Inc. C232085 0812. 2012

[45] Jiang J, Chan TC, Temenak JJ, Dasch GA, Ching WM, Richards AL. Development of a quantitative real-time polymerase chain reaction assay specific for Orientia tsutsugamushi. The American Journal of Tropical Medicine and Hygene. 2004;**70**:351-356

[46] Laube I, Zagon J, Broll H. Quantitative determination of commercially relevant species in foods by real-time PCR. International Journal of Food Science and Technology. 2007;**42**:336-341. DOI: 10.1111/j.1365-2621.2006.01249.x

[47] Jonker KM, Tilburg JJ, Hagele GH, de Boer E. Species identification in meat products using real-time PCR. Food Additives Contaminants Part A Chemical Analysis Control Exposure and Risk Assessment. 2008;**25**:527-533. DOI: 10.1080/02652030701584041.

[48] Nygren J, Svanvik N, Kubista M. The interactions between the fluorescentdyethiazole orange and DNA. Biopolymers. 1998;**46**:39-51. DOI: 10.1002/(SICI)1097-0282(199807)46:1<39:AID-BIP4>3.0.CO;2-Z

[49] Kumar A, Kumar R, Sharma B, Gokulakrishnan P, Mendiratta S, Sharma D. Identification of species origin of meat and meat products on the DNA basis: A review. Critical Review in Food Science and Nutrtion. 2013;**5**:1340-1351. DOI: 10.1080/10408398.2012.693978

[50] Fraga D, Meulia T, Fenster S. Real-Time PCR, Dalam. In: Current Protocols Essential Laboratory Techniques. Hoboken: John Wiley & Sons; 2008. p. 75

[51] Dalmasso A, Fontanella E, Piatti P, Civera T, Rosati S, Bottero MT. A multiplex PCR assay for the identification of animal species in feedstuffs. Molecular and Cellular Probes. 2004;**18**:81-87. DOI: 10.1016/j.mcp.2003.09.006

[52] Yanita IW, Sudjadi Rohman A. Detection of rat meat adulteration in meat ball formulations employing real time PCR. Asian Journal of Animal Sciences. 2015;**9**:460-465. DOI: 10.3923/ajas.2015.460.465

[53] Sudjadi Wardani HS, Sepminarti T, Rohman A. Analysis of porcine gelatin DNA in commercial capsule shell using real-time polymerase chain reaction for halal authentication. International Journal of Food Properties. 2016;**19**:2127-2134. DOI: 10.1080/10942912.2015.1110164

[54] Shehata HR, Li J, Chen S, Redda H, Cheng S, Tabujara N, Li H, Warriner K, Hanner R. Droplet digital polymerase chain reaction (ddPCR) assays integrated with an internal control for quantification of bovine, porcine, chicken and turkey species in food and feed. PLoS One. 2017;**12**:e0182872. DOI: 10.1371/journal.pone.0182872

[55] Shabani H, Mehdizadeh M, Mousavi SM, Dezfouli EA, Solgi T, Khodaverdi M, Rabiei M, Rastegar H, Alebouyeh M. Halal authenticity of gelatin using species-specific PCR. Food Chemistry. 2015;**184**:203-206. DOI: 10.1016/j.foodchem.2015.02.140

[56] Lahiff S, Glennon M, O'Brien L, Lyng J, Smith T, Maher M, Shilton N. Species specific PCR for the identification of ovine, porcine and chicken species in meat and bone meal (MBM). Molecular and Cellular Probes. 2001;**15**:27-35

[57] Ali ME, Razzak MA, Hamid SB, Rahman MM, Amin MA, Rashid NR, Asing. Multiplex PCR assay for the detection of five meat species forbidden in Islamic foods. Food Chemistry. 2015;**177**:214-224. DOI: 10.1016/j.foodchem.2014.12.098

[58] Farouk AE, Batcha MF, Greiner R, Salleh HM, Salleh MR, Sirajudin AR. The use of a molecular technique for the detection of porcine ingredients in the Malaysian food market. Saudi Medical Journal. 2016;**27**:1397-1400

[59] Fitrianingsih. Optimization of DNA isolation method from processed meat as a basic step for pork contamination detection. [Thesis]. Faculty of Animal Science, Gadjah Mada University; 2013