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Multiplex PCR Method of Detecting Pork to Guarantee Halal Status in Meat Processed Products

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ABSTRACT

One of the halal parameters of food is must be free from pork among from the basic ingredients, additives and the manufacturing process. The aim of this study was ensure the halal status in the form of presence or absence of pork in processed meat product (meatballs and sausages) in traditional markets in Pandeglang Regency. PCR multiplex was a PCR technique that used several primers together in one reaction to amplify several target sequence. The genes often used as markers of animal or meat types include cytochrome b (cyt b), the existence of sequence variations in cyt b causes these genes are widely used as markers to distinguish material from different types of animals. The results showed that the cyt b gene proved successful in amplified DNA from beef and pork with different fragment lengths in the DNA mix of the 2 types of livestock in one reaction so that 2 DNA bands formed with different lengths according to the length of the fragment from each animal. From the results of research showed beef and pork control there was two fragments such as 274 bp for beef and 389 bp for pork. Multiplex PCR testing on meatball samples showed that the meatballs were tested 100% positive containing beef and 0% containing pork DNA. While testing on sausage products, there were one sausage brand that showed the results of DNA amplification of 398pb, which means the product was positive containing pork.

Keywords: Meatball, cyt b, DNA, PCR, sausage

ABSTRAK

Salah satu parameter status halal bahan pangan tersebut harus bebas dari kandungan babi baik dari bahan dasar, bahan tambahan maupun proses pembuatannya. Tujuan dari penelitian ini adalah memastikan status kehalalan berupa ada tidaknya kandungan babi pada bahan pangan olahan daging (bakso dan sosis) yang beredar di pasar tradisional di wilayah Kabupaten Pandeglang. Multipleks PCR adalah suatu teknik PCR dengan menggunakan beberapa primer secara bersama-sama dalam satu reaksi untuk amplifikasi beberapa daerah target. Gen-gen yang paling sering digunakan sebagai penanda jenis hewan atau daging diantaranya adalah cytochrome b (cyt b), adanya variasi urutan pada cyt b menyebabkan gen ini banyak digunakan sebagai penanda untuk membedakan material yang berasal dari jenis hewan yang berbeda. Hasil penelitian menunjukan bahwa gen cyt b terbukti berhasil mengamplifikasinya DNA dari hewan sapi dan babi dengan panjang fragmen yang berbeda-beda dalam campuran DNA (DNA mix) dari 2 jenis hewan ternak tersebut dalam satu reaksi sehingga terbentuk 2 pita DNA dengan panjang berbeda sesuai denga panjang fragmen masing-masing hewan. Dari hasil penelitian terhadap kontrol daging sapi dan babi menghasilkan dua fragmen yaitu sebesar 274 pb untuk sapi dan 389 pb untuk babi. Pengujian multipleks PCR pada sampel bakso menunjukan bakso yang diujikan kandungan DNA nya 100% positif mengandung sapi dan 0% mengandung babi. Sedangkan pengujian pada produk sosis terdapat satu merk sosis yang menunjukan hasil ampifikasi DNA sebesar 398pb yang berarti produk tersebut positif mengandung babi.

Kata kunci: Bakso, sitokrom b, DNA, PCR, sosis

INTRODUCTION

The role of animal protein especially meat, was quite important in order to achieve nutritional worthiness standards. Changes in consumption patterns as well as between people cause animal food needs as primary needs that must be met because it is proven can make the community intelligent, active and productive so that the increase in consumption of animal protein is expected to improve the quality of human resources.

Food safety of animal product certainly cannot be separated from attention. Food safety was define as the conditions and efforts needed to prevent food from possible biological, chemical, physical and other contaminants that can harm and endanger human health (Government Regulation No. 28/2004). The government has tried to protect consumers with a variety of laws and government regulations, but until now falsification of food products, especially processed meat products were still common. Mixing other kind of meats in processed meat products usually to reduced production costs. Many cases of fraud and contamination with the use of materials that are not suitable for consumption and not halal. Contamination of these materials can occur at an early or final stage of production and some was accidental. The problem arises if the mixing of meat product used the type of meat that may not be consumed by certain people related to religion and culture.

Detection and identification methods for meat and processed products continue to be developed as an effort to protect consumers and implement food labeling. Specific DNA amplification techniques for each animal type in food safety and halal can be used for verification, certification and monitoring. Some researchers have used the *cytochrome b* (cyt b) gene to distinguish material from different types of animals. Gene cytochrome b (cyt b) was mitochondrial DNA which more numerous than the nucleus DNA and its eternal presence. So the use of this gene is expected to enable the success of PCR amplification with the availability of DNA was many times more than nucleus DNA and the extraction results sufficient to detect samples in small amounts. Specific primary use for pork has been carried out by several researchers in recent years (Fatimah 2013), (Hertanto *et al.* 2017), (Marlina *et al*. 2013), (Maryam *et al.* 2015), (Rahmawati *et al.* 2016), (Rohman *et al*. 2017), (Zulfahmi 2015). The genes most commonly used as markers of animal or meat types include cytochrome b (cyt b), 12S and 16S ribosomal RNA subunits and displacement loop (D-loop) regions. Aim of this study was to detect pork contamination in meat processed products in Pandeglang Banten using specific primers derived from cyt b sequences in pigs using the PCR multiplex technique.

MATERIALS AND METHODS

DNA Samples

DNA samples in this study came from meat and meat processed products. The sample consisted of positive controls there was beef and pork, meat processed products in the form of 5 samples meatballs and 5 samples sausage products. All samples was collect from Pandeglang Regency using an accessible source population method then labeled and stored at freezer temperatures until DNA extraction was carried out.

Primer

Primers used for amplification of specific DNA fragments of beef and pork followed Matsunaga *et al*. (1999). The forward primers used for both types of meats are 5-GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA-3 '. The reverse primary sequences used in the study was present in Table 1.

Table 1. Specific Primer Sequences of Gene Cyt b From Beef and Pork

Sample	Reverse (5' – 3')	Amplification Result
Beef *	CTA GAA AAG TGT AAG ACC CGT AAT ATA AG	274 bp
Pork *	GCT GAT AGT TTT GTG ATG ACC GTA	398 bp

Procedure

DNA Isolation and Extraction

DNA isolation and extraction methods in this study came from fresh meat samples and meat processed products using a Rneasy mini kit 50 (QIAGEN).

Testing of Total DNA

Extracted DNA as qualitatively and quantitatively tested by spectrophotometer. DNA samples of 3µl was put into 1.5 mL eppendorf tubes plus 597 µL distillate water. The TE solution (Tris EDTA) used as a blank in the same way as 3 µL of TE solution plus distillate water, then put in a 1.5 mL eppendorf tube. The samples and blanks were spin down for 0.5 minutes, then tested using a spectrophotometer. Testing of DNA doom with UV spectrophotometers at wavelengths of 260 nm and 280 nm. The extracted DNA concentration was calculated from A 260 times the diluent factor and the DNA absorption constant (50 µg/mL).

Amplification of Specific DNA Fragments

Amplification of the *cyt* b gene segment was carry out by the multiplex PCR method. DNA samples of 2 µL were put into a 0.2 mL PCR tube, PCR components were added consisting of 10 pmol forward primers, 5 pmol reverse primers, 200 mL dNTP mix, 1 mM $MgCl_2$, and 0.5 units of T*aq* polymerase enzymes and the buffer. The amplification process was carried out on a GeneAmp® PCR System (Applied Biosystems TM) thermocycler with a temperature of 94 ^oC for 5 minutes, 30 cycles consisting of denaturation of 94 ^oC for 30 seconds, annealing 60 ^oC for 45 seconds, elongation 72 ^oC for 1 minute and Final elongation of 72 ^oC for 5 minutes. After the process was complete, the tube was taken and stored at room temperature or at 4 °C until it will be further analyzed.

Electrophoresis and Visualization of PCR Products

PCR products visualized in agarose gel 1.5% used

electrophoresis techniques. The gel was made from 0.6 gram agarose and 30 ml of heated buffer solution (0.5 × TBE). Agarose solution allowed cool slightly while stirring with a stirrer, then added 2.5 µL ethidium bromide dye. 5 µL of PCR product were dissolved in 1 µL loading dye. Electrophoresis was carried out for 40 minutes at a constant voltage of 100 volts or until the bromtimol blue dye reaches the bottom of the gel. After electrophoresis completed, the gel taken for UV photography.

RESULTS AND DISCUSSION

The quality and quantity of DNA in an organism can be determined by using a spectrophotometer. The principle of this tool was the irradiation of ultraviolet light absorbed by the nucleotides and proteins in solution. Ultraviolet irradiation by nucleotides was maximally achieved at 260 nm wavelength, whereas maximum ultraviolet irradiation by proteins is achieved at 280 nm wavelengths (Tenrilulo, Suryati, Parenrengi and Rosmiat 2017). Based on research that has been done on the level of purity and concentration of DNA from isolation, the data obtained are presented in Table 2.

Table 2. Purity and Concentration of DNA Solution in Beef, Pork, Meatballs and Sausage Samples Using UV-Vis Spectrophotometer

Sample	Purity A260/A280 (nm)	Concentration (µg/mL)
Beef	1.87	558.90
Pork	2.19	610.65
Meatball 1	1.83	11.85
Meatball 2	1.75	21.35
Meatball 3	1.79	18.15
Meatball 4	1.69	12.90
Meatball 5	1.71	8.60
Sausage 1	1.62	22.90
Sausage 2	1.81	45.95
Sausage 3	1.80	21.00
Sausage 4	1.47	7.25
Sausage 5	1.87	13.80

The results of absorbance measurements at a wavelength of 260/280 nm was vary because it was depends on the source of the DNA obtained. DNA extracted from meat had a ratio of absorbance values in the range of 1.8 to 2.0 than DNA extracted from processed products. DNA molecules are said to be pure if the A / 280 ratio ratio of 280 is equal to 1.8-2.0 (Fatchiyah 2011). These results indicated the level of DNA purity from meat is higher than DNA derived from processed products. This is likely caused by the contamination of proteins and other mixtures used in processed products.

Very important to know how much DNA concentration was used in research. The concentration of extracted DNA was varied. This was due to the extracted samples coming from different sources there was meat and meatballs and sausage. According to the KapaBiosystem (2014) recommendation cited by Nugroho *et al.* (2017), the template DNA concentration needed for PCR activities ranged from 10-100 µg/mL whereas according to Maryam (2014) the optimal concentration in PCR amplification at 30 cycles to produce thick bands was a 50 µg/mL concentration both in beef comparison, pork, beef meatball samples and pork meatball samples. In this study showed that the concentration of DNA in beef and pork samples was very high, namely 558.90 and 610.65 µg/mL, because the extracted sample was a fresh meat without any mixture material.

Concentration of DNA extracted from meatballs and sausage was relatively less because in extracted sample there was a mixture of other ingredients such as flour, spices and other ingredients that was not contain DNA. Beside that some treatments in making processed meatball and sausage products can cause difficulties during DNA isolation and extraction. Important stages that affect the difficulty of DNA isolation from meatballs and sausages were (1) mechanical processing including chopping and grinding meat on meatballs and sausages can cause changes in particle size, shape and composition of meat constituent proteins so that it affects the state of cells which contain DNA, (2) heating treatment with high temperature and pressure such as boiling in boiling water (100 ^{o}C) the process besides causing denatured protein also affects the stability of DNA, (3) mixing of additives in meatballs and sausages in the form of flour and spices the other.

Matsunaga *et al.* (1999) showed that DNA can be isolated from meat that has been heated at temperatures of 100 ^{o}C and 120 ^{o}C for 30 minutes. Nuraini (2004) succeeded in isolating and amplifying DNA from body organs and processed meat products such as meatballs, sausages, corned beef, beef jerky and shredded, or it can be said that heating does not damage DNA (Nuraini 2004). Martín *et al.* (2007) succeeded in amplifying mitochondrial DNA in 12SrRNA regions in DNA samples of cat, dog and mouse meat heated at 120 ^{o}C for 50 minutes, 110 ^{o}C for 120 minutes and 133 ^{o}C at 300 kPa pressure for 20 minutes. According to Kesmen *et al.* (2007), DNA amplification was not influenced by adding a spicies or cooking proccess. Based on purity and concentration data extracted and isolated DNA this study as a whole is good and can be used for the amplification process

The used of the cyt b gene had extensively carried out in research for the identification of types of meat (Di Pinto *et al*. 2005, Asensio 2007, Hsieh *et al*. 2007, Tanabe *et al.* 2007, and Lin *et al*. 2008). Besides using cyt b as a biological marker in species identification, 12S rRNA genes (Fajardo *et al.* 2006) and 16S rDNA (Rastogi *et al.* 2007) can be used, all of which are mitochondrial DNA genes.

The cyt b DNA fragment amplification process used specific primers refers to Matsunaga *et al.* (1999), with the same forward primers used for all types of animals namely 5 '-GAC CTC CCA GCT CCA TCA AAC ATC TCA TGA TGA TGA AA-3', and reverse primer for cattle that was 5 "-CTA GAA AAG TGT AAG ACC CGT AAT ATA AG-3 "and pigs is 5" -GCT GAT AGT AGA TTT GTG ATG ACC

GTA-3 ". The results of the amplification of cyt b fragments in meatball samples in determining the presence or absence of pig DNA contamination was presented in Figure 1.

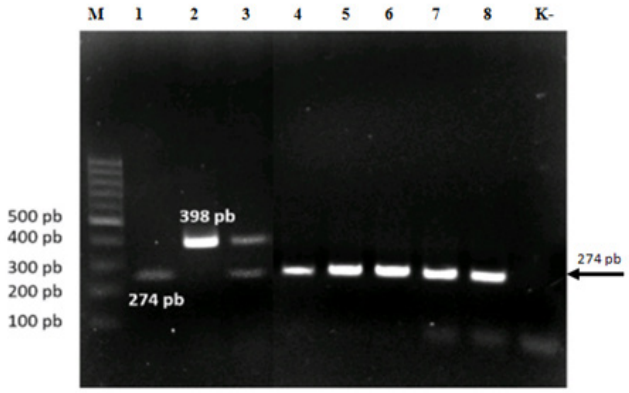

Figure 1. Visualization of results of amplification of DNA cyt b fragments of meatball samples on agarose gel 1.5%, (M) Marker 100 bp, beef control sample (1), pork control (2), beef and pork mixture control (3), meatball samples (4 -8) and K-negative controls in the form (water)

From Figure 1 showed that the results of cyt b gene amplification in agarose gel produce two different fragments there were 274 bp DNA fragments of beef and 398 bp DNA fragments of pork this was according to Primasari (2011) and Irine (2013) who succeeded in amplification of beef and pork fragments of 274 bp and 398 bp respectively. DNA analysis results showed that for all meatballs samples 100% positive contained beef DNA and negative contained pork DNA. This indicates that beef meatball samples in the Pandeglang was safe for consumption and there was no pork DNA contamination. While the identification analysis of sausage products can be seen in Figure 2.

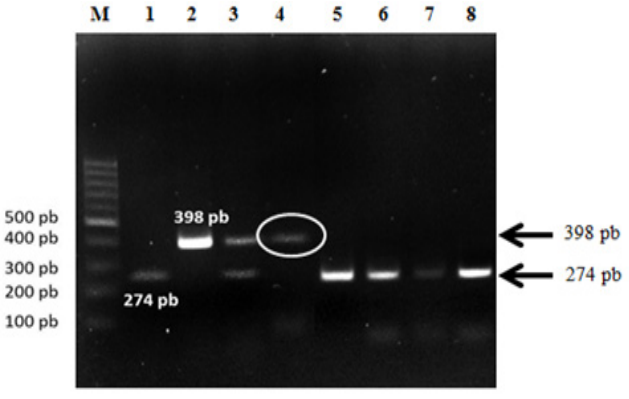

Figure 2. Visualization of the results of amplification of DNA cyt b sausage samples on agarose gel1.5%, (M) Marker 100 bp, beef control sample (1), pork control (2), beef and pork mix control (3), sausage sample (4-8)

Based on Figure 2. showed that the amplification of cyt b genes of beef and pork in sausage samples was successfully proven by visualization on agarose gels (1.5%) where DNA bands were formed in all sausage samples analyzed. The results of the analysis showed that there was sausage product contained pork DNA where 398 bp of fragments were seen in the product test, namely sausage sample no.4 so that it was confirmed to be positive containing pork DNA. While the results of cyt b gene amplification of product samples number 5, 6, 7, and 8 produce DNA bands with fragments of 274 bp which indicate that the sausage product sausage was 100% positive contain beef DNA. The results of this study indicated that meat processed products sausages in Pandeglang Regency was not safe from contamination of pork DNA even though the sausage samples used in this study at packaging printed out MUI halal label and distribution permit number issued by BPOM.

Fontanesi *et al.* (2008) and Langen *et al.* (2010) detected pork based on the sex of pigs in the market. They found that pork that was marketed both fresh and processed could be identified and could even be known whether the pig was male or female. Detection method with specific primers showed positive results in pigs and did not amplify the same genes from other types of animals namely cows, chickens, turkeys and several types of microorganism that often contaminate meat.

Kesmen *et al.* (2007) used species-specific primers to detect meat species in sausage products with a variety of meat mixtures (horses, cows, donkeys and pigs) and showed results that could be detected up to a level of 1% by producing different amplicons. Specific primers design was obtained from ATPase8 (ATP synthase subunit 8), ATPase6 (ATP synthase subunit 6), ND2 = NADH (dehydrogenase subunits 2) and ND5 = NADH (dehydrogenase subunit 5). The results of research to identify species with specific promers have also been developed by Martin *et al.* (2007) which was able to detect the presence of beef, goat and lamb up to the level of 1.0% in a mixture of flour and other vegetable materials. The study also explained that cooking to temperatures of 120 ^{o}C for up to 50 minutes could still be used for PCR reactions with specific primers designed from the 12S rRNA gene.

Detection and identification techniques of animal origin, especially in food products, very important because they was related to health, economy and religion. Safe and halal meat was a major concern, especially for the majority Muslim community of Indonesia. Detection and identification methods of meat types usually used protein analysis, but this method had a weakness it was only can be done in fresh meat sample, required a large sample and very low accuracy when done on processed meat.

The used of multiplex PCR and the superiority of cyt b gene variation in this study proved to be successful in detected and identified the type or source of meat quickly, precisely and accurately. The primers was used one forward primer and 2 reverse primers arranged in a specific area in each animal. The specificity of the cyt b gene was proven by the amplification of DNA from beef and pork with different fragment lengths and the DNA mix of the 2

types of livestock can be amplified in one reaction so that 2 DNA bands formed with different lengths according to the fragment length each animal. So the advantages of multiplex PCR techniques that can save time and cost of analysis because to detected samples with different DNA sources can be done in one reaction so this methode can be more efficient.

CONCLUSION

Amplification of the gene cyt b in beef, pork and meat processed products resulted in two different DNA fragments there was 274 bp for beef DNA and 398 bp for pork. Other types contamination of meat in processed meat products could still analyzed for DNA used multiplex PCR technique even though the processed meat products had undergone mechanical treatment, temperature treatment and also the addition of other food additive materials. The results of DNA analysis of processed meat products in the Pandenglang showed that for five meatball samples that observed was all samples negative containing pork DNA and 100% contained beef DNA. As for the sausage product tested, it was found one sample of five tested samples proved to be positive containing pork DNA, which was shown from the DNA band resulted from electrophoresis visualization on agarose gel 1.5. this study indicated that meat processed products sausages in Pandeglang Regency was not safe yet from contamination of pork DNA.

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