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Specific Species DNA Amplification as a Halal Authentication Method in Sausage Based on Genetic Markers

(Amplifikasi DNA Spesifik Spesies sebagai metode autentikasi halal pada sosis berdasarkan penanda genetic)

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ABSTRACT. Sausage is a processed meat food that is susceptible to counterfeiting by mixing non-halal meat, so a valid, fast and cheap halal authentication method is needed for sausages. This study aims to determine the specific species genes of Cytochrome-b in target DNA amplification as a halal authentication method in sausages. In this study, the samples used were beef sausage, chicken sausage, and pork sausage. The stages of this research consisted of DNA purification, measuring DNA quality, amplifying object DNA using Cytochrome-b, electrophoresis, and visualization of the gel documentation. The DNA purification results from beef sausage, chicken sausage, and pork sausage respectively obtained concentrations of 3 (ng/µl), 2.6 (ng/µl), and 2.8 (ng/µl), and purity of 1.15, 0.76, and 0.88. Meanwhile, DNA amplification produced fragments with lengths for beef sausage, chicken sausage, and pork sausage, namely the Cytochrome-b gene 274 bp, 227 bp, and 398 bp. Based on this study, it's concluded that the Cytochrome-b genes could amplify target DNA from beef sausage, chicken sausage, and pork sausage so that they could be used as gene markers for authenticating halal sausages.

Keywords: marker gene, halal food, halal sausage, PCR, processed meat

ABSTRAK. Sosis merupakan makanan olahan daging yang rentan terhadap pemalsuan dengan mencampurkan daging non halal, sehingga diperlukan metode autentikasi halal yang valid, cepat dan murah untuk sosis. Penelitian ini bertujuan untuk mengetahui gen spesies spesifik Cytochrome-b pada amplifikasi DNA target sebagai metode autentikasi halal pada sosis. Dalam penelitian ini, sampel yang digunakan adalah sosis sapi, sosis ayam, dan sosis babi. Tahapan penelitian ini terdiri dari pemurnian DNA menggunakan Quick-DNA™ Plus Kits, Zymo Research, pengukuran kualitas DNA, amplifikasi pada DNA target menggunakan gen Cytochrome-b, elektroforesis, dan visualisasi pada gel dokumentasi. Hasil pemurnian DNA sosis sapi, sosis ayam, dan sosis babi masing-masing diperoleh konsentrasi 3 (ng/µl), 2,6 (ng/µl), dan 2,8 (ng/µl), dan kemurnian 1,15, 0,76, dan 0,88. Sedangkan amplifikasi DNA menghasilkan fragmen dengan panjang untuk sosis sapi, sosis ayam, dan sosis babi yaitu gen Cytochrome-b 274 bp, 227 bp, dan 398 bp. Berdasarkan penelitian ini, disimpulkan bahwa gen Cytochrome-b dapat mengamplifikasi DNA target dari sosis sapi, sosis ayam, dan sosis babi sehingga dapat digunakan sebagai penanda gen untuk autentikasi sosis halal.

Kata kunci: gen penanda, makanan halal, sosis halal, PCR, olahan daging

INTRODUCTION

Indonesia is a country with the largest Muslim population in the world. As Muslims, people in Indonesia should consume halal food and beverages. They are prohibited from consuming haram food and beverages. In Islamic law, the prohibition of food or beverages can be caused by the type of food and drink itself or by the process. One of the forbidden foods is pork. The prohibition of pork based on the Word of Allah SWT in the Al-Quran Surah Al-Ma'idah verse 3 and verse 88, An-Nahl verse 115, Al-Baqarah verse 173, and Al-Anam: 145 (Syukriya & Faridah, 2019; Zulaekah & Kusumawati, 2015).

Sausage is a processed meat food whose popularity is on the rise in Indonesia in recent years. Research (Santoso *et al*., 2018) reported an average consumption increase of 4.46% per year, and (Hidayat, 2020) also reported a rise in sausage uptake in the modern retail market by 9.9% experienced by PT Belfoods Indonesia. With a population of 270.20 million people and a population increase of 3.26% (BPS, 2021), sausage has a substantial market share. Some irresponsible people sometimes use this situation to get high profits by mixing non-halal meat in sausages with a halal label or making food with non-halal ingredients without giving information that the product is not halal as happened with meatballs (Balia *et al*., 2014; Fitrilia *et al*., 2017).

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Currently, several halal authentication methods have been developed. According to (Hossain *et al*., 2020), halal authentication techniques can be carried out using protein-based species identification such as Chromatography, ELISA, SDS-PAGE, and Spectroscopy, as well as DNA-based such as conventional PCR, Real-Time PCR, and DNA Biosensors. The results of similar research have been reported. For example, protein profile analysis using SDS-PAGE (Hermanto & Meutia, 2009), fat profile analysis using Gas Chromatography (GC) Fourier Transform Infra-Red Spectroscopy Second Derivative (FTIR-2D) (Prabawati & Fajriati, 2018), and testing using the Enzyme-Linked Immunosorbent Assay (ELISA) method (Cahyaningsari *et al*., 2019). However, this method still has some drawbacks. SDS-PAGE electrophoresis is still challenging to characterize protein in beef and pork sausage because some of the protein in sausage has been degraded (Hermanto & Meutia, 2009). Gas Chromatography (GC) and Fourier Transform Infra-Red Spectroscopy Second Derivative (FTIR-2D) have only been proven in the characterization of beef and pork fat in meat, but not in their processed products (Prabawati & Fajriati, 2018). The ELISA method is proven to distinguish beef and pork meatballs (Cahyaningsari *et al*., 2019) but has not been tested on sausages.

Therefore, it is still necessary to develop a halal sausage authentication method that has high validity. One of the halal sausage authentication methods that may be applied is halal authentication based on genetic markers using PCR. Polymerase Chain Reaction (PCR) is a scientific technique in molecular biology to duplicate one or several sequences of DNA fragments into many copies, which produces thousands to millions of copies of a particular DNA sequence (Joshi & Deshpande, 2011). The PCR method is a method that can identify living species based on their DNA sequences. Species identification using the PCR method can be made only by using sample fragments without seeing the complete form of the living thing to be identified (Hebert *et al*., 2003). This method has been proven to be used for species authentication for both meats and processed meat products (Cahyaningsari *et al*., 2019; Perestam *et al*., 2017).

The use of various PCR methods for halal authentication on meat and its derivative products has been widely carried out and has given good results (Akbar *et al*., 2021; Arini *et al*., 2018; Barakat *et al*., 2014; Cahyaningsari *et al*., 2019; Hutami, Suprayatmi, *et al*., 2018; Matsunaga *et al*., 1999; Maulani *et al*., 2020; Nakanishi *et al*., 2021; Raharjo *et al*., 2012; Sugiana *et al*., 2018; Thangsunan *et al*., 2021). One method of halal authentication on meat that is simple and fast is using PCR with the target gene Cytochrome-b (Matsunaga *et al*., 1999; Park *et al*., 2013). According to (Matsunaga *et al*., 1999), Cytochrome-b gene PCR can still amplify DNA from meat cooked at a temperature of 100-120 ^{0}C for 30 minutes.

In contrast to previous studies, where PCR of the Cytochrome-b gene was used to identify species in boiled, in this study, amplification of the Cytochrome-b gene was used to identify species in sausages. Research on the amplification of the Cytochrome-b gene in sausages is critical because previous studies showed that DNA purification from beef sausage produced DNA with low concentrations and purity (Purnomo *et al*., 2021). This study aims to determine the suitability of species-specific primers for Cytochrome-b genes in target DNA amplification as a halal authentication method in sausages.

MATERIALS AND METHODS

Materials

This research was carried out at the Integrated Biotechnology Laboratory, Faculty of Animal Science, Hasanuddin University, Makassar. As much as 50 mg of each sample of beef sausage, chicken sausage, and pork sausage was DNA purified using Quick-DNA™ Plus Kits, Zymo Research. The purification method is carried out referring to the company's procedures.

Table 1. Information of primer sequence

Gene	Primer	Primers Sequence	Source
Cytochrome-b	Forward	5GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA-3	Matsunaga *et al*., 1999
	Reverse cow	5-CTAGAAAAGTGTAAGACCCGTAATATAAG-3	
	Reverse chicken	R: 5-AAGATACAGATGAAGAAGAATGAGGCG-3	
	Reserve pig	5-GCTGATAGTAGATTTGTGATGACCGTA-3	

DNA Quality and Quantity

DNA quality examination was carried out using a UV VIS Spectrophotometer, Thermo Spectronic, and Genesys 10S UV VIS spectrophotometer at a wavelength of 160 and 180 nm. The concentration and purity of DNA are calculated according to the formula (Maftuchah *et al*., 2014; Sambrook *et al*., 1989):
DNA concentration = A260 × 50 × (diluent factor)
DNA Purity = A260/A280

DNA Amplification

DNA amplification using Cytochrome-b gene with primer sequence are presented in Table 1. DNA amplification was performed using the DreamTaq™ Hot Start Green PCR Master Mix from Thermo Fisher Scientific on the PCR machine SensoQuest, Germany, under initial denaturation conditions at 94 ^{0}C x 2 minutes, followed by the subsequent 35 cycles of denaturation at 94 ^{0}C x 2 minutes each. Forty-five seconds, the annealing temperature is 60 ^{0}C x 30 seconds, followed by extension: 72 ^{0}C x 60 seconds, ending with one final extension cycle at 72 ^{0}C for 5 minutes. The amplified DNA was then electrophoresed on 1.5% agarose gel with 1x TBE buffer (89 mM Tris, 89 mM boric acid, two mM Na2EDTA) containing 100 ng/ml ethidium bromide at a voltage of 104 for 45 minutes, then visualized on a UV transilluminator (gel documentation system) GBOX from Syngene.

RESULTS AND DISCUSSION

Quality of purified DNA from sausage

The results of DNA concentration and DNA purity from beef sausage, chicken sausage, and pork sausage using a spectrophotometer are presented in Table 2.

Cyt-b gene

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55
Primer          GACCTCCCAG CTCCATCAAA CATCTCATCT TGATGAAA-- ---------- ----------
Cow             GACCTTCCAG CCCCATCAAA CATTTCATCA TGATGAAATT TCGGTTCCCT CCTGGGAATC
Chicken         GACCTCCCAG CCCCATCCAA CATCTCTGCT TGATGAAATT TCGGCTCCCT ATTAGCAGTC
Pig             GACCTCCCAG CCCCCTCAAA CATCTCATCA TGATGAAACT TCGGTTCCCT CTTAGGCATC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
Primer          ---------- ---------- ---------- ---------- ---------- ----------
Cow             TGCCTAATCC TACAAATCCT CACAGGCCTA TTCCTAGCAA TACACTACAC ATCCGACACA
Chicken         TGCCTCATGA CCCAAATCCT CACCGGCCTA CTACTAGCCA TGCACTACAC AGCAGACACA
Pig             TGCCTAATCT TGCAAATCCT AACAGGCCTG TTCTTAGCAA TACATTACAC ATCAGACACA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
Primer          ---------- ---------- ---------- ---------- ---------- ----------
Cow             ACAACAGCAT TCTCCTCTGT TACCCATATC TGCCGAGACG TGAACTACGG CTGAATCATC
Chicken         TCCCTAGCCT TCTCCTCCGT AGCCCACACT TGCCGGAACG TACAATACGG CTGACTCATC
Pig             ACAACAGCTT TCTCATCAGT TACACACATC TGTCGAGACG TAAATTACGG ATGAGTTATT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
Primer          ---------- ---------- ---------- ---------- ---------- ----------
Cow             CGATACATAC ACGCAAACGG AGCTTCAATG TTTTTTATCT GCTTATATAT GCACGTAGGA
Chicken         CGGAATCTCC ACGCAAACGG CGCCTCATTC TTCTTCATCT GTATCTT--- ----------
Pig             CGCTACCTAC ATGCAAACGG AGCATCCATG TTCTTTATTT GCCTATTCAT CCACGTAGGC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   245        255        265        275        285        295
Primer          ---------- ---------- ---------- ---------- ---------- ----------
Cow             CGAGGCTTAT ATTACGGGTC TTACACTTTT CTAG------ ---------- ----------
Chicken         ---------- ---------- ---------- ---------- ---------- ----------
Pig             CGAGGCCTAT ACTACGGATC CTATATATTC CTAGAAACAT GAAACATTGG AGTAGTCCTA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345        355
Primer          ---------- ---------- ---------- ---------- ---------- ----------
Cow             ---------- ---------- ---------- ---------- ---------- ----------
Chicken         ---------- ---------- ---------- ---------- ---------- ----------
Pig             CTATTTACCG TTATAGCAAC AGCCTTCATA GGCTACGTCC TGCCCTGAGG ACAAATATCA

                ....|....| ....|....| ....|....| ....|...
                   365        375        385        395
Primer          ---------- ---------- ---------- --------
Cow             ---------- ---------- ---------- --------
Chicken         ---------- ---------- ---------- --------
Pig             TTCTGAGGAG CTACGGTCAT CACAAATCTA CTATCAGC
```

Figure 1. Primary DNA sequence and target region of the Cytochrome-b gene

Table 2. Concentration and purity of purified DNA from beef, chicken, and pork sausage.

Sample	DNA Concentration (ng/µl)	DNA Purity
Beef sausage	3	1.15
Chicken sausage	2.6	0.76
Pork sausage	2.8	0.88

Source: this research

Amplification of Sausage DNA

The results of DNA amplification of beef, chicken, and pork sausage using Cytochrome-b gene primers were obtained, as shown in Figure 1 below.

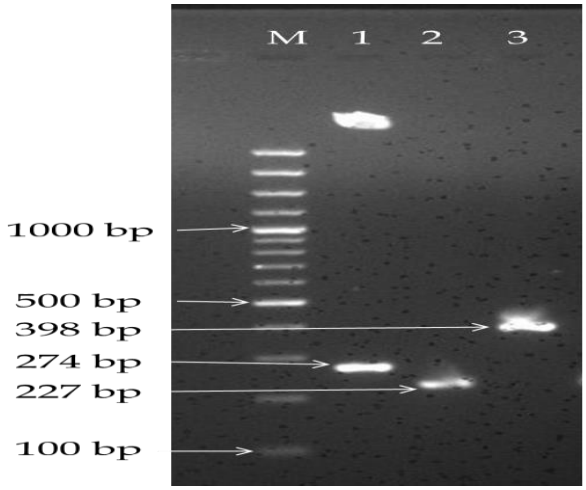

Figure 2. Electrophoresis results of Cytochrome-b gene amplification products from three types of sausages. Note: M: DNA Leader 100bp, 1: beef sausage, 2: chicken sausage, and 3: pork sausage.

DISCUSSION

The results of measuring the concentration and purity of DNA using a spectrophotometer at wavelengths of 260 and 280 nm obtained DNA concentrations from beef sausage, chicken sausage, and pork sausage 3 ng/µl, chicken sausage 2.6 ng/µl, and 2.8 ng/µl respectively. The purity of DNA purified from beef sausage, chicken sausage, and pork sausage were respectively 1.15, 0.78, and 0.88. The results of this study are not much different from the results of (Purnomo *et al*., 2021) that DNA purification from beef sausage produced DNA with an average concentration of 2.75 ng/µl with a purity of 0.99, and (Popping *et al*., 2010) which states that the purification of processed food products ranges from 0.1-5 ng/100 mg sample. However, the results of DNA purification in this study were much lower than the results of DNA purification from the liver and muscle tissue of 40-70 ng/100 mg sample (Popping *et al*., 2010), 483,3 ng/µl meat sample, 486,6 ng/µl brain samples and 1950 ng/µl liver samples (Biase *et al*., 2002), and 177,20 ng/µl fresh beef sample, 410,18 ng/µl fresh chicken sample, and 303,90 ng/µl fresh pork sample (Hutami, Bisyri, *et al*., 2018). The purity of DNA obtained in this study is also lower than the study results of (Biase *et al*., 2002; Hutami, Suprayatmi, *et al*., 2018) in the fresh tissue of the animal body, which is around 1.80-2.24.

The DNA concentration obtained in this study was shallow. According to (Seipp *et al*., 2010) that for DNA amplification using PCR a template with a concentration of more than 10 ng/µl is required with a purity of 1.8-2. The low concentration and purity of DNA obtained in this study may be due to the small amount of meat used in making sausages. Meanwhile, a minimum of 35% ground beef is used (BSN, 2015). In addition, the cooking process and the addition of acid preservatives may be other causes why the concentration and purity of purified DNA from sausages are low. The results of the study (Nur & Suryani, 2011) found that sausages sold in the city of Jogjakarta contained 83.35-211.29 mg/kg Nitrite.

Although the concentration and purity of the purified DNA from sausages were low, the amplification was carried out using the Cytochrome-b gene primer designed (Matsunaga *et al*., 1999) still gives good results. Amplification of the cytochrome-b gene in the DNA of beef sausage, chicken sausage and pork sausage produced DNA fragments with lengths of 274 bp, 227 bp and 398 bp respectively. Based on the results of gene bank data searches, the primers used in this study succeeded in amplifying the target DNA as seen in Figure 1. The DNA fragments resulting from the amplification can also be visualized on an agarose gel and can show differences in the length of DNA from each sample as seen in Figure 2. The results of this study are in line with research (Matsunaga *et al*., 1999; Park *et al*., 2013) on DNA amplification from commercial meat samples and meat boiled at 100-120oC for 30 minutes. In addition, the Cytochrome-b gene can also be used in the examination of adulteration of meat used for making sausages.

Cytochrome-b gene primers can be used as halal authentication markers in sausages. These primers can provide valid, easy-to-do, fast, and relatively inexpensive results. To get the results of halal authentication on sausages, it only takes about 5-6 hours, very few samples are needed, and

high-quality DNA is not required. The results of this study are also a correction from the results of previous studies, which stated that for PCR, it is better to use template DNA with a concentration of more than 10 ng/µl with a purity of 1.8-2 (Seipp *et al*., 2010).

PCR is an in-vitro DNA amplification method by enzymatic method at an exponential rate which involves a repeated cycle process consisting of denaturation, annealing, and extension (Giasuddin, 1995). One of the PCR components is a primer (Giasuddin, 1995; Joshi & Deshpande, 2011). Primers are short artificial DNA strands, with no more than 50 nucleotides (usually 18-25 bp), complementary to each other at the beginning and end of the DNA to be amplified (Joshi & Deshpande, 2011). Annealing is the process of attaching the primer to the DNA template, usually at a temperature of 45-60 ^{0}C depending on the primer (Giasuddin, 1995; Joshi & Deshpande, 2011). Incorrect annealing temperature can cause the primers not to adhere or stick to the DNA template randomly (Joshi & Deshpande, 2011). PCR has been used for various purposes, such as for the diagnosis of various human diseases, research, and experiments, genetic fingerprinting, and genealogical examination. It is also used in the analysis of specimens from archaeological material, the detection of genetic mutations in humans, the detection of pathogenic microbes, the examination of natural bacteria that cannot be cultured, and other laboratory research (Giasuddin, 1995; Joshi & Deshpande, 2011).

CONCLUSIONS AND SUGGESTION

Conclusion

Based on the results and discussion, it can be concluded that Cytochrome-b genes can amplify the target DNA in the DNA template from sausages well. Cytochrome-b gene can be used as a marker in authenticating halal sausages.

Suggestion

To increase the validity of the use of Cytochrome-b genes as markers and ensure the halalness of sausages traded in the market, it is recommended to test the halal authentication at various levels of non-halal meat mixture in sausages to determine the primary accuracy level of the Cytochrome-b gene as a marker gene. Regularly carry out halal authentication for sausages in modern and traditional markets to ensure the safety of Muslim consumers from non-halal sausages.

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