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Detection of species substitution in raw, cooked, and processed meats utilizing multiplex-PCR assay

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ABSTRACT The rise of beef consumption in Indonesia opens an opportunity for "rogue" suppliers to mix beef with other meat species that are relatively cheaper, such as pork, chicken, etc. The aim of this study was to identify pig and chicken meat in raw, cooked, and processed meat products using multiplex-PCR of mitochondrial DNA *Cytochrome b* gene, which is maternally inherited and widely used for forensic studies. A total of 90 samples–33 raw meats, 33 cooked meats, and 24 meatballs–were used in this study. Each sample was extracted to obtain the DNA genome and this was then amplified using multiplex-PCR. The PCR products were visualized using 2% agarose gel electrophoresis. The results showed that species contained in raw, cooked, and processed meat samples could be identified as indicated by DNA bands at 398, 274, 227, and 157 bp for pig, cattle, chicken, and goat species respectively. This study concluded that species substitution in raw, cooked, and processed meats could be detected using the *Cytochrome b* gene as a genetic marker through multiplex-PCR assay.

KEYWORDS *Cytochrome b*, meatball, meat substitution, multiplex-PCR

1. Introduction

In recent years, the development of halal tourism makes Indonesia one of the main halal tourist markets along with other Southeast Asian and Middle East countries (Mohsin et al. 2016). In 2012, Dinar Standard reported that 66% of the Muslim tourists considered halal food the most important issue while traveling in foreign countries. Therefore, providing halal culinary for Muslim tourists is urgently needed to develop halal tourism. Currently, halal food has become a concern since many consumers, both Muslims and non-Muslims, choose a lot of halal-labeled food, therefore many European food industries are interested to invest in this field (van der Spiegel et al. 2012).

At this present, Indonesia has regulations related to halal and food security listed in Law number 33 of 2014 concerning assurance of halal products. The fact is in food processing industries, especially meat, cases of mixing and cheating in other forms are common to increase profits (Nakyinsige et al. 2012). Adulteration on meat products to increase profits is usually done by mixing meat that is cheaper and has similar characteristics such as pork and chicken (Fajardo et al. 2008; Ali et al. 2012).

One of the accurate ways and cheap methods is DNA-based assays, particularly polymerase chain reaction which is well known as PCR (Haider et al. 2012). The PCR is an identification method by specifically amplifying specific DNA fragments in certain locations such as the mitochondrial DNA *Cytochrome b*, *12S rRNA*, *ND2* and *ND5* genes (Chisholm et al. 2005; Kesmen et al. 2009; Cahyadi et al. 2020). They have been widely used for evolutionary and forensic studies due to abundantly available in the cells, maternally inherited, no recombination occurred, and high mutation rate among species (Lockley and Bardsley 2000). Those properties make them very useful biomarker to construct phylogenetic tree and species discrimination including in animal-based products (Cahyadi et al. 2020).

Multiplex-PCR is a kind of conventional PCR developed to amplify several DNA targets simultaneously in one reaction tube using multiple primer pairs. This technique is much more efficient compared to species-specific PCR, PCR-RFLP, or real-time PCR because it does not need more reagents or current technology (Cahyadi et al. 2020). Previous study reported that utilizing mitochondrial *Cytochrome b* gene can detect goat, chicken, cattle, sheep, pig and horse genetic materials in raw and cooked meats (Matsunaga et al. 1999). In addition, pork contamination can be identified in processed meat product containing only 1% pork (Novianty et al. 2017) and multiplex-

PCR using *Cytochrome b* gene can detect the species existence in foods up to 0.002 ng/µL genomic DNA (Hossain et al. 2017). This study evaluated the efficiency of multiplex-PCR *Cytochrome b* gene to detect species in the sample designed the same as those found in the market. Therefore, the purpose of this study was to detect species substitution in raw, cooked, and processed meat products utilizing multiplex-PCR with mitochondrial DNA *Cytochrome b* gene.

2. Materials and Methods

2.1. Sample collection

Four species, bovine, porcine, chicken, and goat, have been used as DNA template sources. Their meats were obtained from the traditional market of Pasar Gede in Surakarta City. Raw meat samples with good physical quality were chosen and cooked meat samples were obtained by boiling 1000 mg of meat with boiling water at 100 °C for 30 min. The meat mixture was made by equal proportion for each species, except for pig which was added 5% or 5 g per 100 g meat. The detail of meat samples and their mixtures are presented in Supplementary Table 1.

2.2. Meatball making process

The meat product identified in this study was self-made meatballs based on a predetermined composition and percentage of meat (Supplementary Table 2). Meats were ground separately using meat grinders until smooth, and then it was mixed with flour and spices that have been graded. The mixture was then formed into spheres using a spoon and boiled in boiling water at 100 °C for 30 min until the meatballs float (Bintoro 2008). Moreover, samples were drained and then separately placed and labeled according to their composition.

2.3. DNA extraction

A total of 90 DNA samples extracted from 33 raw meats, 33 cooked meats, and 24 meatballs were used in this study. The DNA extraction procedure was carried out according to the Quick-DNA Universal Kit procedure (Zymo Research Ltd., USA). A total of 25 mg of raw, cooked, and processed meats from each sample were used to harvest the DNA genome from those samples. Extracted DNA genome was visualized using 1% agarose gel electrophoresis stained by FloroSafe DNA Stain (1st BASE, Singapore) under the UV light.

2.4. Simplex-, duplex- and multiplex-PCR

The results of DNA extraction were amplified according to the KAPA2G 2X Fast Multiplex Mix PCR Kit procedure (Kapa Biosystems, Inc., USA) by targeting the mitochondrial *Cytochrome b* gene using primers designed by Matsunaga et al. (1999). Primer sets are presented in Table 1. The total PCR volume was 25 µL consisting of 12.5 µL PCR mix (Kapa Biosystems, Inc., USA), 0.5 µL primer for each species, 1 µL DNA template. The ddH_2O was added up to reaching 25 µL. The PCR reaction was carried out on a thermal cycler machine (GeneAmp® PCR System 9700, Singapore) with an initial temperature of denaturation 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Finally, the final extension process at 72 °C for 3 min was applied to complete PCR process. The results of simplex-, duplex- and multiplex-PCR were visualized using 2% agarose gel electrophoresis (Vilber Lourmat Infinity 1100126M, France).

3. Results and Discussion

3.1. Result

The result of simplex PCR is shown in Figure 1. It can be seen that raw meat samples and cooked meat samples can be well visualized on 2% agarose gel under UV light. This process was the first step to determine the specificity and to ensure no cross-contamination among samples. A good simplex PCR showed bright and clear single DNA band which is indicated by 398 bp for pig, 274 bp for bovine, 227 bp for chicken, and 157 bp for goat respectively. In addition, simplex PCR used meatballs as samples were also successfully conducted in this study (Figure 4).

Duplex PCR was done by mixing two different types of meat in order to be identified together. The samples used were also both raw and cooked meats as shown in Figure 2. This result indicated that duplex PCR could be

TABLE 1 Species, *Cytochrome b* gene primers, PCR product size and annealing temperature used in this study.

Species	Nucleotide	PCR product size
Pig (*Sus scrofa*)	F: 5'-GACCTCCCAGCTCCATCAA-ACATCTCATCTTGATGAAA-3'	398 bp
	R: 5'-GCTGATAGTAGATTTGTGA-TGACCGTA-3'	
Cattle (*Bos taurus/Bos indicus*)	F: 5'-GACCTCCCAGCTCCATCAA-ACATCTCATCTTGATGAAA-3'	274 bp
	R: 5'-CTAGAAAAGTGTAAGACC-CGTAATATAAG-3'	
Chicken (*Gallus gallus*)	F: 5'-GACCTCCCAGCTCCATCAA-ACATCTCATCTTGATGAAA-3'	227 bp
	R: 5'-AAGATACAGATGAAGAAG-AATGAGGCG-3'	
Goat (*Capra hircus*)	F: 5'-GACCTCCCAGCTCCATCAA-ACATCTCATCTTGATGAAA-3'	157 bp
	R: 5'-CTCGACAAATGTGAGTTAC-AGAGGGA-3'	

F is forward primer; R is reverse primer, bp is base pair.

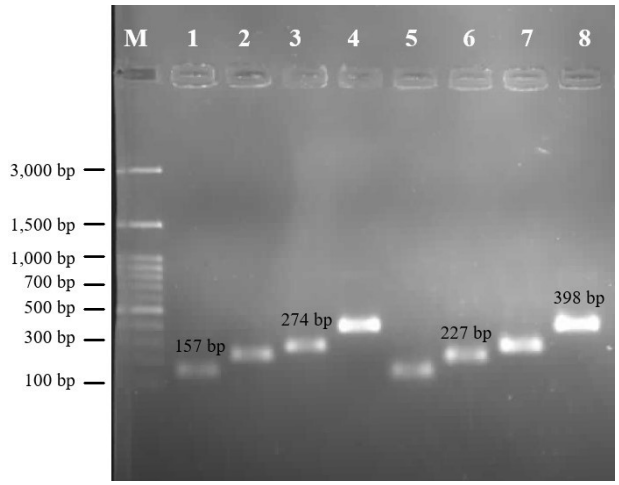

FIGURE 1 Simplex PCR with *Cytochrome b* gene as a target. M is 100 bp marker ladder; 1, 2, 3, 4 are simplex PCR products of raw meat for goat, chicken, cattle, and pig, respectively; and 5, 6, 7, 8 are simplex PCR products of cooked meat for goat, chicken, cattle, and pig, respectively.

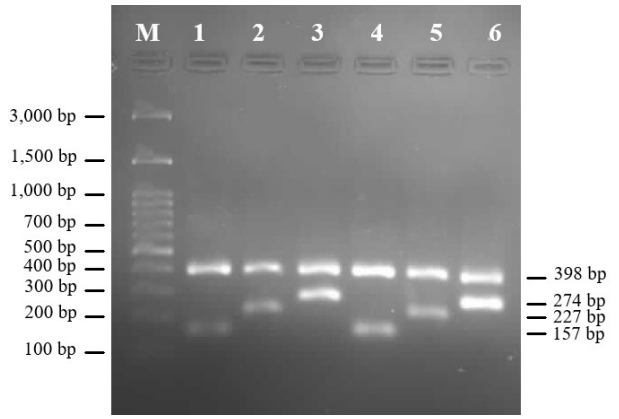

FIGURE 2 Duplex-PCR products of raw and cooked meat. M is 100 bp marker ladder; 1 is PCR product for raw mutton and pork as DNA templates; 2 is PCR product for raw chicken and pork as DNA templates; 3 is PCR product for raw beef and pork as DNA templates; 4 is PCR product for cooked mutton and pork as DNA templates; 5 is PCR product for cooked chicken and pork as DNA templates; 3 is PCR product for cooked beef and pork as DNA templates.

run in both raw and cooked meats. Meat substitution was important to be detected, especially in the sample containing pork and chicken meat.

Identification of samples using multiplex-PCR save costs and time since it can be conducted to detect more than one species only in a tube of PCR mixture. There were twelve sample types which are consistently shown great results in this study. Eight DNA mixture of raw and cooked meats were successfully extracted and amplified using *Cytochrome b* as the target region (Figure 3). In addition, multiplex-PCR was also conducted using meatball as sample and showed promising results (Figure 4). The results indicated that multiplex-PCR was a powerful tool to identify pork and chicken substitution in raw, cooked, and processed beef and mutton which are literally much more expensive meats.

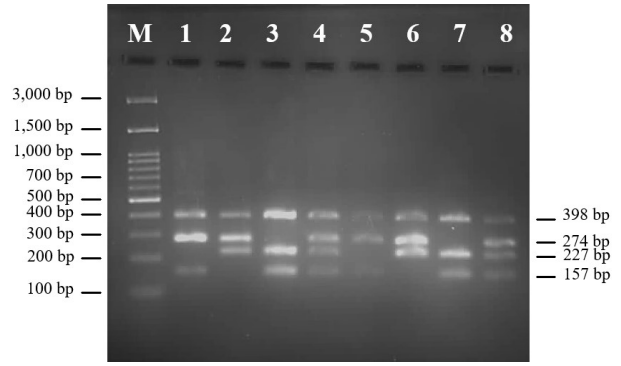

FIGURE 3 Multiplex-PCR products of raw and cooked meat. M is 100 bp marker ladder; 1 is PCR product for raw beef, mutton and pork as DNA templates; 2 is PCR product for raw chicken, beef and pork as DNA templates; 3 is PCR product for raw mutton, chicken and pork as DNA templates; 4 is PCR product for four species as DNA templates; 5 is PCR product for cooked beef, mutton and pork as DNA templates; 6 is PCR product for cooked chicken, beef and pork as DNA templates; 7 is PCR product for cooked mutton, chicken and pork as DNA templates; 8 is PCR product for four species as DNA templates.

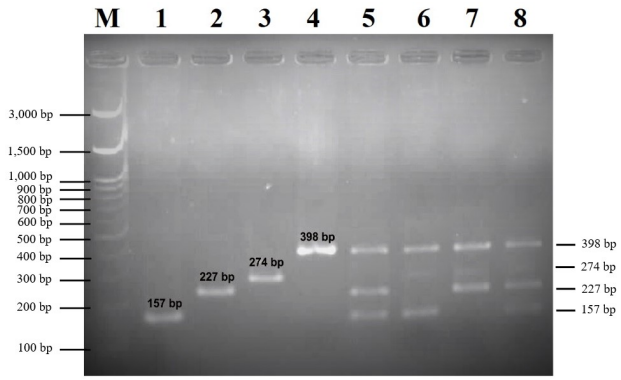

FIGURE 4 Simplex- and multiplex-PCR products of meatball sample (processed meat). M is 100 bp marker ladder; 1, 2, 3, 4 are simplex PCR products of raw meat for goat, chicken, cattle, and pig, respectively; 5, 6, 7, 8 are multiplex PCR products of meatball samples contaminated with pork.

3.2. Discussion

Testing various meats and processed meat products is an important thing to be performed to avoid unfair market competition and efforts to protect consumer rights. Therefore, today many methods are selected and used to be tested to obtain the most effective and efficient way of detecting species substitution in meat-based products. Utilizing PCR assay is proven to be able to identify meat species correctly and accurately even though the meat has been cooked and mixed with other ingredients (Matsunaga et al. 1999; Fajardo et al. 2008; Mane et al. 2012).

This study demonstrated that utilizing multiplex-PCR assay has successfully detected species substitution in raw, cooked, and processed meat products. The mitochondrial DNA *Cytochrome b* gene is widely used as a target gene in detection of genetic materials containing in foods (Mat-

sunaga et al. 1999; Ali et al. 2012, 2015; Nakyinsige et al. 2012; Novianty et al. 2017). Mitochondrial DNA is well-known as circular DNA materials tend to be more resistant towards heat and copy number of mitochondrial DNA are much more than the nucleus DNA since every single cell containing more than one mitochondrion. Therefore, it can be used as DNA marker to check the originality of meat products treated by high temperature and grinding (Fajardo et al. 2008; Ghovvati et al. 2009; Mane et al. 2009; Cahyadi et al. 2020). Like other mitochondrial DNA genes, *Cytochrome* b gene is maternally inherited. It is approximately 1140 bp in length encoding 380 amino acids and commonly used for species determination due to its nucleotide sequence variability (Castresana 2001).

Duplex PCR is done by identifying two different types of meat simultaneously. The samples used were samples of cooked beef, chicken, and mutton, then they were contaminated with pork. All samples were tested with pork as contaminant because pork is a type of forbidden meat by Islam. Halal food authentication is an important matter concerned several countries, especially for Muslims (Murugaiah et al. 2009). Research conducted showed that porcine DNA bands can be clearly seen in each contaminated sample. Therefore, it can be said that PCR method is an accurate and reliable method for detecting species (Ballin et al. 2009; He et al. 2015). Moreover, chicken meat was also successfully detected by this assay. Chicken meat is mostly used to substitute commercially meat-based products to reduce production cost.

Multiplex-PCR was done to test large sample quantities in a short time and minimum cost. It can be carried out to detect more than one species containing in meat products only in single-tube reaction. All species were able to be clearly detected without any cross-contamination using raw, cooked, and processed meat products. However, bovine genetic material was not clearly appeared in multiplex-PCR assay using meatball samples. Low bovine DNA concentration extracted from meatball made from meat mixtures may lead to less bright DNA band. Moreover, concentration of extracted DNA from mixed samples cannot be precisely similar for each species, and high temperature and grinding treatments may cause low DNA genomic harvested and amplicon (Cahyadi et al. 2020). This study used genomic DNA harvested from designed meat samples. Five percent (5%) pork was intentionally added into the sample and it was clearly able to be detected by multiplex-PCR targeting *Cytochrome b* gene. This study attempted to detect species origin of the actual meat product sold in the commercial markets and/or restaurant. Therefore, the sample mixture was designed as closely as possible to those commercially sold. Indeed, rogue sellers substitute costly meats with more than 5% cheaper meats to get more profits. The sensitivity test of multiplex-PCR in this study was very good, except for meatball made from meat mixtures. This study showed positive impacts in detecting pork and chicken meat which are used as main meat substitution sources in meatball production. Substitution of undesirable meats in meatball production cannot be detected by bare eyes, therefore laboratory testing should be carried out (Rohman et al. 2011). Meatball is very popular dish in Southeast Asian Countries especially in Indonesia which is known as the biggest Muslim population in the region. Furthermore, providing halal and safe food is not only for Muslims in Indonesia but also for every single human living in this world. They should be protected to consume undesirable materials in foods.

4. Conclusions

This study proved that multiplex-PCR technique was very powerful tool to detect meat substitution in raw, cooked, and processed meat products. It may be useful to be applied to check commercial meat products in protecting consumers from inauthentic products.

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Authors’ contributions

MC designed most of the experiment, wrote original draft, and edited final version of the manuscript. ITS helped in collecting samples, making meatballs, DNA extraction, and performing PCR. NADF performed data interpretation and wrote some parts of original draft. WB edited final version of the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The author declare that they have no competing interest.

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