

## DETECTION OF PORK CONTAMINATION IN FRESH AND COOKED BEEF USING GENETIC MARKER MITOCHONDRIAL-DNA CYTOCHROME B BY DUPLEX-PCR

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### ABSTRAK

Pemalsuan daging sapi yang dicampur dengan daging babi merupakan salah-satu permasalahan umat Muslim yang sering ditemukan di Indonesia. Penelitian ini bertujuan untuk mendeteksi daging babi pada daging sapi segar dan masak menggunakan marker genetik DNA mitokondria gen *Cytochrome b* (*mt-DNA Cyt b*) dengan *duplex-PCR*. Penelitian ini menggunakan 6 sampel daging segar dan 6 sampel daging yang dimasak. Sampel daging babi diperoleh dari Rumah Potong Babi (RPB), sedangkan sampel daging sapi dibeli dari supermarket di Kota Surakarta. Sampel daging masak disiapkan dengan cara merebus daging pada suhu 100°C selama 30 menit. Kontaminasi daging babi pada daging sapi segar maupun masak dirancang sebesar 0, 1, 5, 10, dan 25%. Selanjutnya, total DNA diisolasi dari sampel tersebut dan *polymerase chain reaction* (PCR) dilakukan menggunakan primer spesifik untuk mengamplifikasi fragmen spesifik masing-masing spesies. Hasil penelitian menunjukkan bahwa total DNA berhasil diekstraksi dari sampel daging sapi, daging babi, dan campuran keduanya baik pada sampel daging segar maupun masak. Selanjutnya, elektroforesis pada agarosa 1,5% menunjukkan bahwa *duplex-PCR mt-DNA Cyt b* berhasil mendeteksi kontaminasi daging babi pada daging sapi segar maupun masak hingga level 1%. Keberadaan daging babi pada sampel ditunjukkan dengan adanya pita DNA sebesar 398 bp. Kesimpulan penelitian ini adalah *duplex-PCR mt-DNA Cyt b* sangat sensitif dalam mendeteksi cemaran daging babi pada daging sapi segar maupun masak.

*Kata kunci: cytochrome b, daging babi, daging sapi, duplex-PCR, kontaminasi*

### ABSTRACT

By mixing with pork, beef adulteration is frequently found in the traditional market that very disturbing Moeslem community in Indonesia. This study was conducted to detect pork contamination in fresh and cooked beef using genetic marker mitochondrial DNA cytochrome b (*mt-DNA Cyt b*) by *duplex-PCR*. A total of twelve samples was used in this study consisting six fresh meat samples and six cooked meat samples, respectively. Those beef and pork were bought from animal slaughterhouse and a supermarket in Surakarta. Cooked samples were prepared by boiling the meats in hot water at 100°C for 30 minutes. We designed pork contamination in beef in the level of 0, 1, 5, 10, 25%, respectively. The DNA genome was extracted and polymerase chain reaction (PCR) was performed using species specific primer to isolate *mt-DNA Cyt b* gene from the samples. The results showed that the DNA genome was successfully extracted from pork, beef, and contaminated meat samples. In addition, visualization of *duplex-PCR* on 1.5% agarose gel was able to detect pork contamination in both fresh and cooked beef up to very small proportion (1%). The existence of pork in beef was indicated with the presence of specific 398 bp DNA band. It can be concluded, *duplex-PCR* of *mt-DNA Cyt b* gene was very sensitive in detection of pork contamination in fresh and cooked beef.

*Keywords: beef, contamination, cytochrome b, duplex-PCR, pork*

## INTRODUCTION

The awareness of Indonesian to the need of animal protein resources such as beef has steadily been increased recently, however, the beef price is highly expensive in Indonesia. On the other hand, pork is one of cheap animal protein resources than can be found easily in the market. These conditions open an opportunity for some peoples to achieve more profits by mixing beef with pork. Due to cheaper in price and relatively similar characteristics, pork is commonly used for beef adulteration. Previous study reported by Ali *et al.* (2012) revealed that contamination of pork in beef and processed-beef products has been found and widely spread in the capital city in Indonesia.

Islam is the major religion in Indonesia, therefore, utilization of pork and pork derivates are prohibited especially in food for Moslem consumer (Said *et al.*, 2011). According to Islam point of view, pig is one of haram thing that clearly described in holy Al-Qur'an surah Al-Baqarah verse number 173. Therefore, scientific approach is urgently needed to detect contamination of pork and pork derivate to protect Muslim consumer in Indonesia from undesired components in food, especially in beef.

The effort to detect pork contamination in beef has been developed since decades ago. Polymerase chain reaction (PCR) is one of molecular biology approaches which is used to authenticate and to detect species origin in meat by amplifying species specific sequence in the genome such as fragment of mt-DNA Cyt b gene (Girish *et al.*, 2005). Cyt b gene is a gene that frequently used for phylogenetic studies. The primer of Cyt b gene has been designed to amplify specific site for various species for example mammals, birds, amphibian, reptile, fish, insects, and spider (Kocher *et al.*, 1989).

Species detection in meat based on deoxyribonucleic acid (DNA) using PCR has successfully been performed to identify DNA originated from fresh meat and processed meat products (Aida *et al.*, 2005). Multiplex-PCR, a variant of PCR approaches that often used for species authentication, is a molecular technique consisted of two or more loci which is simultaneously performed in one PCR reaction. This method has often been applied to analyze DNA deletion, mutation, and polymorphism (Henegariu *et al.*, 1997). Matsunaga *et al.* (1999) reported that multiplex-PCR has successfully been applied for species identification in raw and

cooked meats. In Indonesia, the use of mt-DNA Cyt b gen to detect species origin in raw meat and meat products has been done. Nuraini *et al.* (2012) and Irine *et al.* (2013) reported that dog, cat, tiger, and rat can be detected using partial sequence of mt-DNA Cyt b gene as a biomarker. Here, we performed both simplex-PCR and duplex-PCR to amplify two target fragments from beef and pork samples. Therefore, the aim of this study was to detect pork contamination in fresh and cooked beef using genetic marker mt-DNA Cyt b by duplex-PCR.

## MATERIALS AND METHODS

### Samples Preparation

Both pork and beef samples were obtained from commercial sources. The meats samples were labeled to make sure that all samples were separated each other and then all meats were stored in the refrigerator until used. Beef samples were contaminated with pork in the ratio 100:0, 75:25, 90:10, 95:5, and 0:100% (beef:pork). Those rations were also applied for cooked samples. For cooked meats, 1000 mg samples were prepared and boiled in water at 100°C for 30 minutes.

### Extraction of DNA

The DNA genome was prepared from cattle and pig by following Genomic DNA Mini Kit for animal tissue protocol (Geneaid Biotech Ltd., Taiwan). A total of 30 mg meat tissue was weighed and transferred to a 1.5 mL microcentrifuge tube, and then the meat was grinded to a pulp using micropestle provided by the kit. Total of 200 µL of GT buffer was added to the tube and homogenized by grinding. Then, 20 µL of proteinase K was also added to the mixture and then the mixture was incubated at 60°C for 30 minutes. Furthermore, 200 µL of GBT buffer was added into the tube and incubated at 60°C for at least 20 minutes to ensure the lysate is clear. For the next step, 200 µL of absolute ethanol was added to the lysate then immediately shaken vigorously for 10 seconds. The mixture was transferred to the GD column placed in a 2 mL collection tube and it was centrifuged at 14.000 rpm for 2 minutes. After that, 2 mL collection tube was discarded and the GD column was transferred to a new 2 mL collection tube. Furthermore, 400 µL of W1 buffer was added to the GD column and centrifuged at 14.000 rpm for

30 seconds, and then the flow-through in the 2 mL collection tube was discarded. The GD column was placed back in the 2 mL collection tube and added 600  $\mu$ L of wash buffer to the GD column for centrifugation at 14.000 rpm for 30 seconds. The flow-through was discarded and then GD column was placed back in the 2 mL collection tube for final centrifugation at 14.000 rpm for 3 minutes to dry the column matrix. In addition, dried GD column was transferred to a clean 1.5 mL microcentrifuge tube and then 100  $\mu$ L of pre-heated elution buffer was added to the center of the column matrix until completely absorbed. The final step, the mixture was centrifuged at 14.000 rpm for 30 second to elute the purified DNA. Then, the DNA genome was visualized by 1% electrophoresis agarose gel using Gel Document (Vilber Lourmat Infinity 1100126M, France) and it is ready to be used for next analysis.

### Simplex- and duplex-PCR

The PCR amplification was conducted in total volume of 25  $\mu$ L containing 12.5  $\mu$ L 2X KAPA2G Fast Multiplex Mix (Kapa Biosystems, Inc., United States), 0,5  $\mu$ L each primer (10  $\mu$ M), 1  $\mu$ L DNA template, and ddH<sub>2</sub>O. To isolate DNA target, three primers which are designed by Matsunaga *et al.* (1999) were used in this study. Those primers were universal forward primer (5'-GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA-3') and reverse primers for bovine (5'-CTAGAAAAGTGTAAGACCCGTAATATAAG3'), and reverse primer for pig (5'-GCTGATAGTAGATTTGTGATGACCGTA-3'). The PCR reaction was carried out using GeneAmp<sup>®</sup> PCR System 9700 (Singapore) as follows: initial denaturation at 95°C for 3 minutes, and folled by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds. To make sure that PCR reaction completed, final extension was applied at 72°C for 1 minute. The simplex- and duplex-PCR were viewed in the agarose gel 1.5% under the UV light using Gel Document (Vilber Lourmat Infinity 1100126M, France).

## RESULTS AND DISCUSSION

### The DNA Extraction

Pork and beef samples were bought from animal slaughterhouse and supermarket in Surakarta, Central Java. A total of 12 samples was designed and used for DNA isolation in this study. In particular, they were six raw meat samples and

six cooked meat samples, respectively. The pork contamination in the beef samples was designed as much as 0, 1, 5, 10, and 25%. The result showed that DNA genome was successfully extracted from both raw meat and cooked meat samples (Figure 1). The principle of genome isolation is extracted the DNA genome from other components of the cells (Muladno, 2012). The cooked meat samples were also obtained. The treatment to the meats did not affect the extraction of DNA genome. Previous studies reported that DNA genome can be extracted from cooked meats such as sausages, cold cut and leisure meat food (Hou *et al.*, 2015; Matsunaga *et al.*, 1999; Nakyinsige *et al.*, 2012; Zhang, 2013). However, the quality of DNA genome isolated from raw meats was better than DNA genome isolated from cooked meats. Figure 1 presents the intencity of the DNA genome isolated from raw meat was clearer than cooked meats under the UV light. Andree *et al.* (2004) reported that heat and physical treatments on the meat products can decrease the quality of DNA genome.

### Detection of Pork Contamination in Raw and Cooked Beef

Both simplex- and duplex-PCR were conducted in this study. Simplex-PCR was applied to optimize the PCR conditions whereas duplex PCR was carried out to detect pork contamination in raw and cooked meats. The existence of pork in the sample mixtures was indicated by 398 bp of mt-DNA Cyt b. This DNA band is specific for porcine. On the other hand, the existense of bovine species in the meat mixture was indicated by appearance of 274 bp of specific mt-DNA Cyt b site for bovine. The results

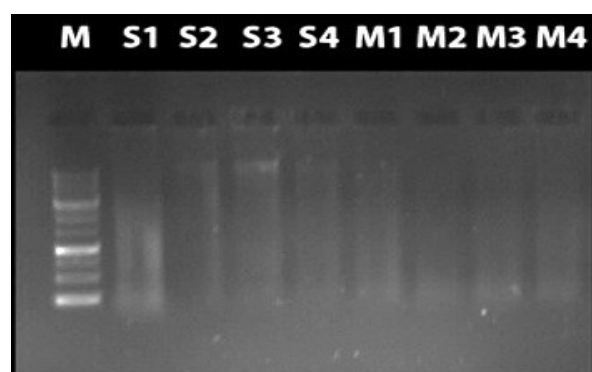


Figure 1. The DNA Genome Extracted from Fresh and Cooked Meats Visualized in 1% Agarose Gel

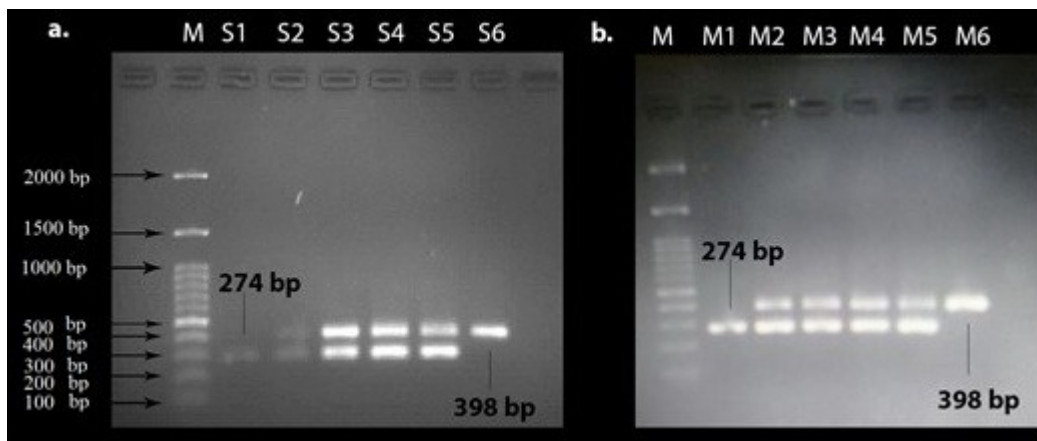


Figure 2. The results of duplex-PCR of mt-DNA Cyt b. Figure 2a is duplex-PCR of fresh meats, M is 100 bp marker ladder, S1 is 100% raw beef, S2 to S5 are beef samples contaminated with 25, 10, 5, 1% pork, and S6 is 100% pork. Figure 2b is duplex-PCR of cooked meats, M is 100 bp marker ladder, M1 is 100% cooked beef, M2 to M5 are cooked beef contaminated with 25, 10, 5, 1% pork, and M6 is 100% cooked pork.

of simplex- and duplex-PCR were presented in Figure 2.

This study was successfully detected pork contamination in fresh and cooked beef. The contamination of pork in beef products can be detected until 1% contamination of pork. The treatment to the meat samples was not affected this approach to identify the existence of pork in fresh and cooked beef. Previous study reported that species origin of fresh and cooked meats can be detected by multiplex-PCR of mt-DNA Cyt b. Kumar *et al.* (2015) developed primers of mt-DNA Cyt b gene to authenticate chevon meat (*Capra hircus*) by PCR assay. Furthermore, Matsunaga *et al.* (1999) reported that species contamination can be detected until 8% in the mixtures. Ali *et al.* (2015) developed multiplex PCR assay for the detection of five meat species forbidden in Islamic foods. Their assays have successfully detected existence of five species forbidden in Islamic foods (cat, dog, pig, monkey and rat meats) by designing species-specific primers of mitochondrial ND5, ATPase 6, and cytochrome b genes to amplify 172, 163, 141, 129 and 108 bp DNA fragments from those species, respectively. In Indonesia, Erwanto *et al.* (2014) found the existence of pork in beef meatballs marketed in Yogyakarta by PCR-RFLP method. Currently, Fang and Zhang (2016) successfully detected the existence of mouse in mutton by using TaqMan real-time PCR. In addition,

Rahman *et al.* (2014) developed PCR assay for the assessment of dog meat adulteration in meatballs. Kitpipit *et al.* (2014) developed direct-multiplex PCR assay for meat species identification in food products. They used mt-DNA fragment to amplify specific DNA fragment for pork, lamb/mutton, chicken, ostrich meat, horse meat and beef, respectively. Furthermore, Rojas *et al.* (2010) reported that polymerase chain reaction assay can be used for verifying the labeling of meat and commercial meat products from game birds. This study amplified specific sequences from the mitochondrial D-loop region.

The molecular biology techniques have been utilized for detection of species origin in the food (Zhang, 2013). In Moslem countries, those assays have currently been applied to verify and to authenticate whether a food product is halal or haram. Van der Spiegel *et al.* (2012) reported that the global halal market is increasing, therefore, standardization and certification of halal products can use laboratory analysis such as DNA-based assays which are very sensitive to achieve appropriate assurance of halal food products and proper labelling for consumers and buyers.

## CONCLUSION

Duplex-PCR of mt-DNA Cyt b gene was able to detect pork contamination in fresh and cooked beef. It may be applied to authenticate

commercial meat products marketed in Indonesia.

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