Identification of pork contamination in meatball using genetic marker mitochondrial DNA cytochrome b gene by duplex-PCR

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Abstract. Meat based food products have a big opportunity to mix and adulterated with other meats. Muslim communities are prohibited to consume pork-containing product or other pig derivatives in food. Therefore, the high sensitivity, fast, cheap and accurate approach is needed to detect pig contamination in raw meat and meat-processed product such as meatball. The aim of this study was to identify pork contamination in meatball using genetic marker of mitochondrial DNA cytochrome b gene by duplex-PCR. Samples were prepared and designed by following the proportions 0, 1, 5, 10, 25% of pork in meatballs, respectively. The DNA genome was extracted from meatballs and polymerase chain reaction (PCR) was performed using species specific primer to isolate mt-DNA cytochrome b gene. The results showed that the DNA genome was successfully isolated from pork, beef, and contaminated meatballs. Furthermore, 2% agarose gels was able to visualize of duplex-PCR to identify pork contamination in meatballs up to very small proportion (1%). It can be concluded that duplex-PCR of mt-DNA cytochrome b gene was very sensitive to identify pork contamination in meatball with the presence of specific 398 bp DNA band.

1. Introduction
Along with the growing of meat and dairy product consumptions, food safety supervision and processed products of animal origin also must be increased. Supervision is carried out to prevent food from possible contamination of biological, chemical or other objects that can harm consumers. Food adulteration is a common problem in the food industry [1]. The production of meat-based foods have a high susceptibility to counterfeiting, especially mixing with the meat of wild animals [2]. In Bali there are cases of machines use the same production between pork and beef sausage [3]. The activity is at risk of contamination of pork products processed sausages. Many meatball traders are using pork as a raw material or mixture in their manufactures [4]. Pork is the type of meat which is often mixed into other processed meat products. Pork has a relatively cheaper price, in addition to the color and shape similar to beef, so it is often used as a compound for lowering the price of production and improving the taste [5]. Pork mixing action against the processed products would be very detrimental to the public, this is because the majority of people in Indonesia are Muslims that forbidden to eat pork [6]. The components of food containing pork in food products can be identified through the fat, protein and deoxyribose nucleotida acid (DNA). The technology polymerase chain reaction (PCR) can be used to detect any species with amplification techniques specific DNA fragments [7]. Meat species detection methods such
as PCR-based DNA is able to detect DNA that has been degraded by the heating process during food processing [8]. The use of PCR technology can detect pig DNA in samples of fresh meat and processed meat [9]. Previous study reported that the gene cytochrome b using PCR technology has a high sensitivity to detect pig DNA. The use of gene cytochrome b with duplex-PCR method was able to identify the contamination of pork in fresh and cooked beef until the level of 1% [10]. Cytochrome b gene is a DNA fragment that is specific. Some researchers reported that cytochrome b gene can be used as a marker of DNA types of meat in the identification of certain types of meat [11]. Animal DNA can also be detected using DNA sequences from the mitochondrial cytochrome b gene as a biomarker. Based on these descriptions mentioned above, duplex-PCR of mitochondrial cytochrome b gene should be done to detect contamination of pork in processed meat product such as meatball.

2. Methods
2.1 Samples Preparation
Sample preparation was conducting by mixing the beef and pork that has been flavored meatballs. Meatball was made by following published method [12]. The sample was designed as follows 0, 1, 5, 10, 25, 100% of pork in beef meatball, respectively. Firstly, sample was grinded and mixed with other ingredients until homogenous. Then, meatballs were boiled at 100°C for 30 minutes. Samples meatballs were cooked and then placed in a 1.5 ml tube to be analyzed in the next step, namely the isolation of DNA.

2.2 Isolation of DNA
The DNA genome was isolated from meatballs by following Quick-DNA™ Universal Kit (Zymo Research). A total of 25 mg meatball was weighed and transferred to a 1.5 ml microcentrifuge tube, and a solution of 95 µl aquabidest (ddH2O), 95 µl Solid tissue buffer (blue) and 10 µl proteinase K were added. The mixture was vortexed and incubated at 55°C until tissue dissolved. Insoluble debris should be removed by centrifugation at 14.000 x g for 1 minute. Furthermore, 200 µl aqueous supernatant was transferred to a clean tube and 400 µl genomic binding buffer was added to the supernatant and then it was mixed thoroughly. The mixture was transferred to a Zymo-Spin™ IIC-XL Column in a collection tube and it was centrifuged at 14.000 x g for 1 minute and then the flow through in the collection tube was discarded. Next, 400 µl DNA pre-wash buffer was added to the column in a new collection tube and it was centrifuged for 1 minute and then the flow through in the collection tube was discarded. Furthermore, 700 µl g-DNA wash buffer was added and centrifuged for 1 minute and then the flow through in the collection tube was discarded. In addition, 200 µl g-DNA wash buffer was added and centrifuged for 1 minute, and then the flow through in the collection tube was discarded. The mixture was transferred to a clean microcentrifuge tube. To elute the DNA, 50 µl DNA elution buffer was added and incubated for 5 minutes, and then it was centrifuged for 1 minute. Then, the DNA genome was visualized by 1% electrophoresis agarose gel using gel document (Vilber Lourmat Infinity 1100126M, France) and it was ready to be used for next analysis.

2.3 Duplex-PCR
The PCR amplification was conducted in total volume of 25 µL containing 12.5 µl 2X KAPA2G Fast Multiplex Mix (Kapa Biosystems, Inc., United States), 1 µl each primer (10 µM), 1 µl DNA template, and 8.5 µl ddH2O. Three primers which are designed by Matsunaga were used in this study to isolate DNA target [13]. Those primers were universal forward primer (5’GACCTCCCCAGCTCATCAAACATCTCATCT TGATGAAA-3’) and reverse primers for bovine (5’-CTAGAAAGTGTAAGACCGTAAATATAA G3’), and reverse primer for pig (5’-GCTGATAGTATTTGTGATGACCCTTA-3’). The PCR reaction was carried out using GeneAmp® PCR System 9700 (Singapore) as follows: initial denaturation at 95°C for 3 minutes, and followed 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 3
minute. The *duplex*-PCR results were viewed in the agarose gel 2% under the UV light using Gel Document (Vilber Lourmat Infinity 1100126M, France).

3. Results and Discussion

3.1 The DNA Isolation

Pork and beef samples were bought from traditional market in Surakarta, Central Java. A total of 6 samples was designed and used for DNA isolation in this study. The pork contamination in the meatballs samples was designed as much as 0, 1, 5, 10 and 25%. The result showed that DNA genome was successfully isolated from meatballs samples. The principle of genome isolation is isolated the DNA genome from other components of the cells [14]. The DNA genome still could be isolated from meat processed product such as meatball. Previous studies reported that DNA genome can be isolated from cooked meats such as sausages, cold cut and leisure meat food [15].

![Figure 1](image-url)

*Figure 1.* Agarose gel electrophoresis of PCR products amplified from beef meatball and pork DNA mixture. M is molecular marker, A is comparison between beef and pork in the mixture (100% beef : 0% pork); B is 0% beef : 100% pork; S1 is 75% beef : 25% pork; S2 is 90% beef : 10% pork; S3 is 95% beef : 5% pork; S4 is 99% beef : 1% pork, respectively.

3.2 Detection of Pork Contamination in Meatballs

In figure 1. showed that M is a molecular marker, A is 100% beef meatball, B is 100% pork meatball, S1 is 75% beef meatball mixed with 25% pork, S2 is 90% beef meatball mixed with 10% pork, S3 is 95% beef meatball mixed with 5% pork and S4 is 99% beef meatball mixed with 1% pork. The existence of bovine in the sample mixtures was indicated by 274 bp of mitochondrial DNA Cytochrome b, on the other hand, the existence of pig species in the meat mixture was indicated by appearance of 398 bp of specific mitochondrial DNA Cytochrome b site for porcine. This study successfully identified of pork contamination in meatball. It means the ingredients in meatballs sample did not influence the DNA isolation. The contamination of pork in beef products can be detected until 1% contamination of pork. Additionally, our current results proved that multiplex PCR able to identify pork and chicken in meatballs commercially marketed in Surakarta (*unpublished data*). Previous study also revealed that species contamination can be detected until 8% in the mixtures by multiplex PCR [14]. In addition, multiplex PCR 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 [5]. The existence of pork in beef meatballs was also detected by PCR-RFLP.
method in the meatball shop around Yogyakarta [16]. The result showed that Cytochrome-\(b\) gene using PCR method had a high sensitivity to detect pork DNA. In Moslem countries, those assays have currently been applied to verify and to authenticate whether a food product is halal or haram. 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 [10].

4. Conclusion

Duplex-PCR of mt-DNA cytochrome \(b\) gene successfully identified the pork contamination until the level of 1\% in the meatball. It may be applied to authenticate commercial meatball marketed in Indonesia.

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