Detection of chicken contamination in beef meatball using duplex-PCR Cyt b gene

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Abstract. Beef is one of expensive animal protein sources compared to other meats, on the other hand, chicken is cheap animal protein source. Mixing of chicken into beef meatball is possibly performed to decrease production cost. The aim of this study was to detect chicken contamination in beef meatball using Cytochrome b (Cyt b) gene by duplex-PCR. Sample was designed and prepared as follows, 100% of chicken meatball, 100% of beef meatball and serial level of chicken contaminations in beef meatball (1, 5, 10 and 25%, respectively). Isolation of DNA genome from meatball was according to the guideline of gSYNC™ DNA Extraction Kit for animal tissue. The PCR reaction was carried out using KAPA2G Fast Multiplex Mix. This study found that the DNA genome was successfully extracted. Moreover, chicken contamination in beef meatball was indicated by the presence of 227 bp DNA band on 2% of agarose gels. Current study revealed that duplex-PCR using Cyt b gene as a genetic marker was able to detect chicken contamination in beef meatball until 1% of chicken meat in the sample. It can be effectively used to identify contamination and also authenticate species origin in animal products to protect consumer from undesirable contents in the food.

1. Introduction
Meat is one of animal protein resources which can be processed into various meat products. One of the meat products favored by Indonesian people is meatball [1]. The main raw material from meatball is beef which prices are more expensive than other meats. These conditions trigger meatball producers to mix with other meats from different species. Mixing of chicken into beef meatball is possibly performed to decrease production cost [2], on the other hand, chicken is easily to be found in the market [24]. There are many cases of mixing chicken into beef meatball at different cities in Indonesia to decrease production cost [3] [4] [5]. Therefore, species detection method is required to authenticate species origin in animal products to protect consumer from undesirable contents in the food. Polymerase chain reaction (PCR) is an enzymatic method to multiply exponentially a certain nucleotide sequence specific by in vitro. The PCR method is highly sensitive, so it can be used to amplify the DNA molecule [6]. PCR method has been used for identification of chicken adulteration in different meat products and raw meat [7] [8]. However, substitution of high-cost meats with low-cost meat species is considered fraudulent practices if it is not indicated on the label. Adulteration has been violated consumer protection laws. This regulation creates a consumer protection system containing elements of legal certainty and transparency of information and access to information [9]. Analysis of PCR from specific sequence of mitochondrial DNA is a method that is easy, fast and can be identified for several species at the same time [10], cooking process did not affect to the DNA...
identification [11]. One of the genes in the DNA of mitochondria is Cyt b [12] which has a sequence variation that is used as a marker to distinguish material originating from different animal species [13]. Cyt b have been used to characterized various vertebrates [14], it can be used to identify vertebrates species which contain specific species information [15]. Here, we performed duplex-PCR to amplify two target fragments from beef, chicken and serial level of chicken contaminations in beef meatball sample. Therefore, the aim of this study was to detect chicken contamination in beef meatball using Cyt b gene by duplex-PCR.

2. Methods
2.1 Sample Collection
Beef and chicken were obtain from Pasar Gede traditional market in Surakarta, Central Java. Each sample was labeled to avoid undesireable contamination.

2.2 Meatball Sample Preparation
A total of 30 meatball was designed and prepared. Meatballs were made by grinding the meat with different grinder in order to avoid undesireable contamination. Beef and chicken were mixed with the following serial level of chicken contaminations, 100% of chicken meatball, 100% of beef meatball and serial level of chicken contaminations in beef meatball (1, 5, 10 and 25%, respectively). Contaminated meat were prepared and mixed until homogeneous with flour, salt, pepper and garlic [16] then they were cooked in a boiled water for 5-10 minutes until the meatball rised to the surface.

2.3 Isolation of DNA Genome
Isolation of DNA genome from meatball sample was according to the guideline of gSYNC™ DNA Extraction Kit for animal tissue. 25 mg of each meatballs was weighed and transfered into a 1.5 ml microtube, and then 200 μl of GST buffer and 20 μl of proteinase K was added. The mixture was mixed thoroughly using the vortex and then incubated at 60°C overnight until the sample lysate becomes clear. During incubation, 1200 μl elution buffer (200 μl for each sample) were transfered to a 1.5 ml microtube and heated 60°C. The supernatant was transfered to a new microtube, then 200 μl of GSB buffer was added and vortex thoroughly for 10 seconds until sample and GSB buffer were mixed thoroughly. A total 200 μl of absolut ethanol was added to the sample lysate and mixed immediately by vortex thorougly for 10 seconds. Furthermore, GS column was placed in a 2 ml collection tube, all the mixture were transfered to the GS column then centrifuge at 14,000-16,000 × g for 1 minute. The 2 ml of collection tube containing the flow-through was discarded then the GS column was transfered to a new 2 ml collection tube. For the next step, 400 μl of W1 buffer was added to the GS column and it was centrifuged at 14,000-16,000 × g for 30 seconds then the flow-through was discarded. The GS column was placed back into 2 ml collection tube. 600 μl of wash buffer was added to the GS column, centrifuged at 14,000-16,000 × g then the flow-through was discarded. The GS column was placed back into 2 ml collection tube, centrifuged again for 3 minutes at 14,000-16,000 × g to dry the column matrix. The dried GS column was transfered to a clean 1.5 microtube, and then 100 μl of pre-heated elution buffer was added into center of the column matrix for 3 minutes to make sure the elution buffer was completely absorbed. After that it was centrifuged at 14,000-16,000 × g for 30 seconds to elute purified DNA. The purified DNA was transfered into a clean 1.5 microtube. Finally, purified DNA were stored in refrigerator until used.

2.4 Polymerase Chain Reaction (PCR)
The PCR reaction was conducted using PCR machine (GeneAmp PCR System 9700, Singapore). The total volume of 25 μl containing of 12.5 μl of KAPA2G Fast Multiplex Mix PCR Kit (Kapa Biosystems, Inc., USA), 1 μl of forward primer, 1 μl of reverse primer for bovine and chicken respectively, 1 μl of DNA template and 8.5 μl aquabidest was carried out. Oligonucleotide primers were forward primer (5’-GACCTC-CAGCTCCATCAAACCATCTCATCTTTGATGAAA-3’), reverse primer for chicken (5’-AGATACAGATGAAG-AATGAGGCC-3’) and reverse primer for bovine (5’-CTAGAAAAGTGTAAAGACC-CGTAATATAAG-3’) [17]. Duplex-PCR was carried out in
phases: initial denaturation at 95°C for 3 minutes, 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 and followed by a final extension at 72°C for 3 minutes. Duplex-PCR result was visualized using gel documentation on 2% of agarose gels.

3. Result and Discussion

3.1 Isolation of DNA Genome

The samples were designed and prepared for DNA isolation. Six DNA samples were extracted from meatballs with serial level of chicken contamination. Each DNA samples was extracted from three different meatballs in composites. Specific site for various species was amplified by the primer of Cyt b gene [18]. Mitochondrial DNA has many advantages over nucleus DNA for authenticate species origin in animal product studies. Mitochondrial DNA has several folds of copy number and relatively more abundant in total nucleid acid preparation than nucleus DNA [19]. This study performed that the DNA genome was succesfully isolated from all of meatballs. Isolation of DNA genome did not affect by cooking process. Previous studies reported that DNA genome can be isolated from cooked meats [17]. The presence of DNA was visualized using gel documentation on 1% agarose gel.

3.2 Polymerase Chain Reaction (PCR)

Duplex-PCR was performed to detect chicken contamination in beef meatball. Mitochondrial DNA Cyt b gene from different species were amplified by PCR. Double stranded DNA denaturated to single stranded DNA then take up a secondary structure that is depend on its sequence [20]. Chicken contamination in beef meatball was indicated by the presence of 227 bp DNA band specific for chicken, on the other hand, the existance of bovine species was indicated by the presence of 274 bp of specific mitochondrial DNA Cyt b site for bovine (Figure 1). The presence of DNA was visualized using gel documentation on 2% agarose gel. This study was succesfully detected chicken contamination in beef meatball. The chicken contamination in beef meatball can be detected until 1% of chicken contamination level. The cooking process did not affect the identification of chicken in beef meatballs. Previous study reported that detection of the other species in processed meat products with PCR [21] was succeeded in detecting the presence of a mixture of pork in beef meatballs. Furthermore, previous study successfully identified the species of meat in processed products using the PCR method including cows, goats, sheep, pigs and horse [17]. Duplex-PCR of Cyt b gene was able to identify pork in fresh and cooked beef until the level of 1% contamination [22]. In addition,
PCR methods have been used for identification of meat and meat product [23]. This current study revealed that PCR method may be applied to authenticate species origin in animal products to protect consumer from undesirable contents in meat-based food.

4. Conclusion
Duplex-PCR of mitochondrial DNA Cyt b gene was able to detect chicken contamination in beef meatball in very small proportion in the samples (1%). It may be very useful tools to authenticate species origin in animal products to protect consumer from undesirable contents in the animal products.

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