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Analysis of beef meatball adulteration with wild boar meat using real-time polymerase chain reaction

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Abstract

The price of beef is high in Indonesia, so that some unethical producers try to blend beef with wild boar meat (WBM) in meatball formulation to gain economic profits. This study aims to use real-time polymerase chain reaction (RT-PCR) for identification of WBA in meatball formulation. The specific primers used are designed using NCBI software and subjected to BLAST procedure. The candidate primers are tested for specificity study using several DNAs from fresh meat of some species, including pork having similar characteristics with WBM. The developed method is also validated by determining several parameters of linearity, sensitivity, precision and efficiency. The results showed that primer CytbAG3A at specific annealing temperature can amplify specifically DNA from WBM with acceptable efficiency (105%). The detection limit of WBM DNA is 48 pg corresponding to 3% of WBM in beef meatball formulation. The developed method is used for analysis of marketed meatballs from Yogyakarta (representative for non-wild boar area), Kalimantan (as representative region of wild boar area) and Timor Leste (representative of other country and wild boar area). RT-PCR using CytbAG3A primer is accurate and reliable method for analysis of WBM in meatball in order to support halal authentication of food products.

Introduction

Indonesian is the largest Muslim community in the world. Indonesian Regulation (Indonesian Act 33, 2014) stipulated that any products (food, cosmetics, personal care, pharmaceuticals) spreading in Indonesia must be certified halal. Due to development in food science and technology, some products can be added intentionally or unintentionally with non-halal components such as pork, wild boar meat and porcine gelatin (Mursyidi, 2013). In order to succeed the implementation of this act, some scientist developed and proposed some detection techniques for identification of non-halal components in the products.

Meatball or known as bakso in Indonesia is one of the favourite meat products due to its nutritional sources of protein for human consumption, its appreciated flavour and taste (Rohman *et al*., 2011; Soares *et al*., 2014). Meat commonly used for meatball preparation is beef, however, due to the high price and low availability of beef, some meatball producers try to blend beef with lower priced meat such as pork and wild boar meat (WBM). Wild boar *(Sus scrofa)* or known celeng or Babi hutan in Indonesia is found in some forest of Kalimantan, Sumatera, and Sulawesi (Republic of Indonesia). The adulteration of WBM into beef meatball can be considered from several aspects of public health, fair trade, and religious consideration, because certain religions like islam prohibited their followers to consume any products containing WBM (Rohman and Che Man, 2012; Rahmati *et al*., 2016).

Some methods have been used for halal authentication, mostly for analysis of pig derivatives as reviewed by Rohman and Che Man (2012). FTIR spectroscopy combined with multivariate calibration is one of the reported method used for analysis of WBM in meatball formulation (Guntarti *et al*., 2015). However, calibration technique is influenced by some factors like derivative treatment, spectral scatter, and even sample presentation methods. On the other hand, the position of FTIR spectral peaks are influenced by lipid contents and moisture contents which make band shipment, and as consequence, the precision is difficult to attain (Rahmati *et al*., 2016). Based on this fact, polymerase chain reaction (PCR) is taken as method of choice for meat identification (Kumar *et al*., 2013). PCR is DNA-based method. Analytical techniques based on DNA identification has advantages. DNA is stable, even toward high temperatures, available in most organism and allows to extract exact information from the identical source regardless of the tissues of origin (Lockley and

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Bardsley, 2000).

PCR techniques are the most reported methods for meat analysis, especially for identification of pork and porcine gelatin in food and pharmaceutical products for halal analysis (Maryam *et al*., 2016; Rahmawati *et al*., 2016; Sudjadi *et al*., 2016). PCR-restriction fragment length polymorphism (RFLP) and PCR specific primer is used by two group of researchers for identification and differentiation of wild boar from pork (Fajardo et al., 2008; Abd. Muthalib *et al*., 2012). RFLP is suitable for the identification of even degraded DNA, but the use of PCR-RFLP technique in large scale food traceability program is cost-effective (Singh and Neelam, 2011), therefore real-time PCR is used for tackling this obstacle. RT- PCR is a powerful technique used to quantify the nucleic acids data in real time, while the reaction proceeds (Nakyinsige *et al*., 2012). In this study, real-time PCR using new specific primers using NCBI software and subjected to BLAST procedure is used for identification of WBM DNA in meatball formulation.

Materials and Methods

Preparation of wild boar-beef meatball formulation and dna extraction

Meatball Formulation was prepared based on a formula described by Purnomo and Rahadiyan (2008). Both raw wild boar and beef were cleaned, sliced, and ground individually. Forty five grams of grounded beef and wild boar was mixed with concentrations of wild boar of 0, 3, 5, 10, 85 and 100% wt/wt in meatball. Furthermore, it is made by emulsifying 90% of meat, then added with 10% of starch and mixed vigorously with salt and certain ingredients (garlich powder, cumin powder, chopped onion and black pepper). The meat and all other ingredients were blended by vigorous mixing and the emulsified homogenous meat mixture was shaped into ball. The meatball is then cooked in boiling water (100°C) for 10-20 min. Pure beef meatball was prepared as a negative control and pure wild boar meatball was used as positive control. For DNA isolation, all samples, raw meats (wild boar, beef, pork, chicken, and goat) and meatball formulation were grounded. Isolation of DNA was performed using DNA isolation Kit K1820-01 (Invitrogen, life technologies, California, USA) according to procedure from Invitrogen .

Real time PCR amplification

Amplification of wild boar DNA on variety of raw meat and meatball formulation was done using SsoFast®Evagreen®SUPERMIXwithtwosetsofprimer of CytbAG3A. The primer is designed using NCBI website using software Primer3Plus and subjected to BLAST procedure. Primer CytbAG3A forward: 5'-AGGCCGGGGCCTATATTA-3' and CytbAG3A reverse: 5'-TCTACGAGGTCTGTTCCGAT-3' (accession number NC_0269921). Amplification of the mitochondrial CytbAG3A primers was performed in a final volume of 20 µL containing 50 ng of extracted DNA, 10 µL of Ssofast Evagreen® supermix (Biorad, USA), 1 µL (10 mmol) of each primer and 6 µL of free nuclease water. The real time PCR step-cycle program: denaturation at 95°C for 30 second, followed by 30 cycles of denaturation at 95°C for 5 second, annealing at 59°C for 30 second and extention at 72°C for 10 second.

Validation of real-time PCR

Test of primer specifity was conducted on five species of raw meats (wild boar, pig, beef, chicken, goat) using optimum annealing temperature. Gradient PCR was carried out using designed primer at various annealing temperature (58–62°C). DNA extracted from meatball formulation containing 100% wild boar meat used as positive control, while DNA from meatball with beef 100% is used as negative control. The sensitivity assay was measured as limit of detection (LoD) of DNA extracted from fresh meat and that extracted from meatball containing 100% wild boar. During LoD determination, the replicates of real-time PCR measurements were made from dilution series of DNA with concentrations of 30,000; 6,000; 1200; 240; 48; 9.6 and 1.92 pg. LoD was determined as the amount of DNA amplified by primer resulting relative fluorescence unit of 100 (Maryam *et al*., 2016). This series is also used for efficiency studies. The repeatability test was performed by computing coefficient variation (CV) value of Ct during real-time amplification using certain concentration of wild boar DNA.

Detection of wild boar adulteration in commercial meatball formulation products

Identification of wild boar adulteration in five commercial meatball sold in Yogyakarta (representative for non-wild boar area) and four commercial meatball formulation derived from south Kalimantan (representative of wild boar area) and Timor Leste (representative of other country and wild boar area). The analysis was done using Primer CytbAG3A with the condition as indicated above.

Results and Discussion

Meat identification in food product is very essential. Real-time PCR is method of choice for meat identification via DNA analysis. Some analyst designed primers which are specific for meat DNA. Primer CytbAG3A is designed using Primer3Plus software and used during PCR analysis. A pair of CytbAG3A has the contents of G-C bases in the last 5 position of the 3' end less than 3. This composition can increase the specific binding at the 3' does not form GC clamp folds (Wang and Seed, 2006). This primer is subjected to validation test (primer specificity, sensitivity, linearity, efficiency and repeatability) (Widyasari *et al*., 2015).

The specificity test of primer CytbAG3A is subjected toward DNA from different meat namely pork, beef, rat's meat, chicken, and goat. These meat are commonly used as meat in meatball. PCR analysis using primer CytbAG3A revealed that the used primer is specific to DNA wild boar, as indicated that primer can amplify wild boar DNA and not amplify other meat DNA. In addition, during PCR analysis, some annealing temperature is optimized. Temperature of 59.0°C is selected as annealing temperature due to its capability to provide the highest intensity compared others (Figure 1). During PCR analysis, the number of cycles is limited to 30 cycles. The primer is also specific to wild boar DNA extracted from meatball using different meat.

The sensitivity of PCR analysis using primer CytbAG3A is performed by diluting a series of DNA from 100% wild boar meatball and that extracted a series concentration of DNA from wild boar meatball formulation. The standard curve revealed that amplification in fresh meat DNA results coefficient of determination (R^2) value for correlation between cycle threshold (Cq) value (y-axis) and log DNA concentration (x-axis) is 0.999 (100% wild boar meatball DNA) and 0.945 (series concentration of DNA from wild boar meatball formulation) with efficiency values (E) of 105.7% (Figure 2.A) and 102.5% (Figure 2.B), respectively. These E values meet the criteria set by Biorad (2006) and Muhammad *et al.* (2015). Based on calibration curve, it is reported that LoD values of DNA extracted from 100% wild boar meatball is 48 pg, corresponding to 3% of wild boar meat in meatball formulation, as shown in the amplification curve in Figure 3. Below 48 pg, there is no amplification or Ct value obtained is higher than intercept value.

The precision of real-time method is evaluated by repeatability test (same analyst, same instrument and with short interval time). The relative

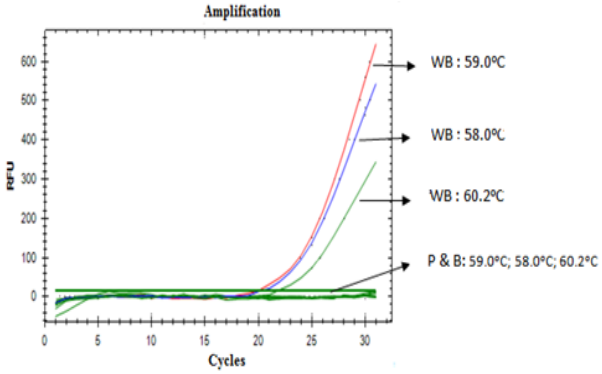

Figure 1. Amplification curve of wild boar using primer CytbAG3A at different annealing temperature. Temperature of 59.0°C is selected as annealing temperature due to its capability to provide the highest intensity. WB (wild boar), P (pork), B (beef)

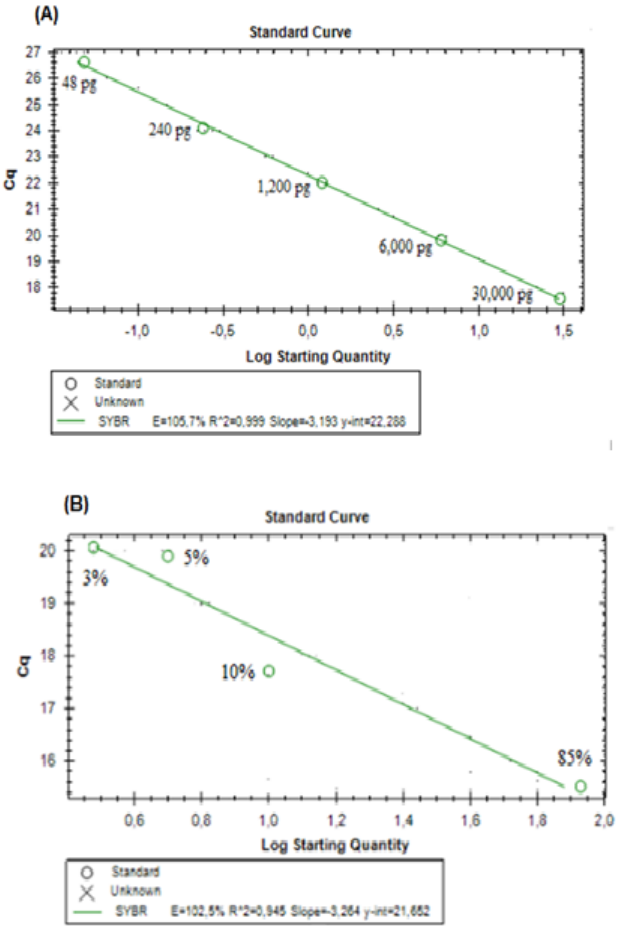

Figure 2. The calibration curve of DNA amplification in 100% wild boar meatball (A) and DNA extracted from wild boar meatball formulation (B)

standard deviation (RSD) value is used to measure repeatability test. The PCR analysis showed positive amplification from all DNA samples containing wild boar meat. Codex Allimentarius commission (CAC, 2010) set maximum RSD values for precision of real-time analysis of 25%. The RSD values obtained are 10.02% (for DNA extracted from 100% wild boar

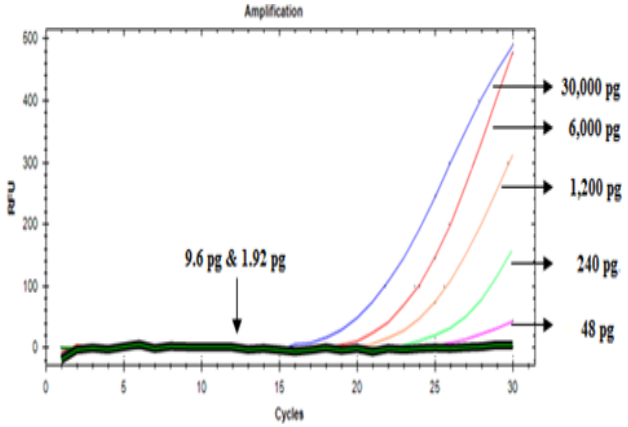

Figure 3. Amplification curve of 5 dilution series using isolate DNA from 100% wild boar meatball for sensitivity assay.

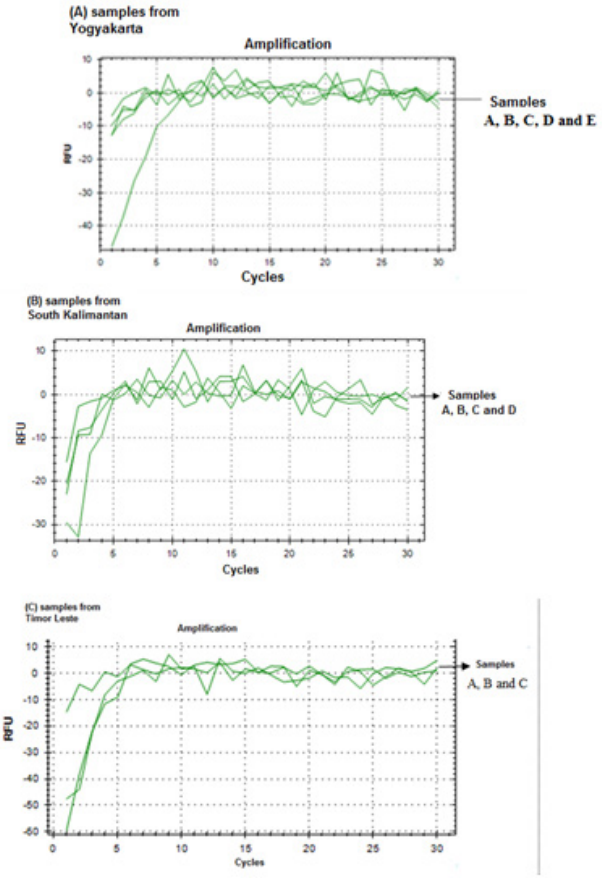

Figure 4. The Amplification curve of commercial meat ball samples obtained from Yogyakarta (A), South Kalimantan (B) and Timor Leste (C).

meatball), and 5.80% for DNA extracted from wild boar meatball formulation lower than that required by CAC. It can be stated that real-time PCR is precise enough for analysis of wild boar DNA in meatball formulation.

The validated method using primer CytbAG3A is then used for analysis of meatball adulteration with wild boar from several region, namely fromYogyakarta (representative for non-wild boar area), South Kalimantan (representative of wild boar area) and Timor Leste (representative of other country and wild boar area). The analysis was done using with the condition as indicated above. There are no amplification peaks of DNA extracted from meatball marketed in Yogyakarta (Figure 4.A), South Kalimantan (B) and Timor Leste (C). Therefore, it can be concluded that the evaluated meatballs do not contain wild boar meat in its formulation.

Conclusion

Real-time PCR using primer CytbAG3A designed from NCBI website has been successfully validated by determining primer specificity, linearity, efficiency, sensitivity and repeatability for analysis of wild boar meat DNA in meatball formulation. The primer is specific for wild boar meatball. The method is fruitfully applied for analysis of commercial meatball in region of Yogyakarta, South Kalimantan and Timor Leste. All evaluated samples do not contain wild boar meat. The developed method is suitable for routine analysis of wild boar meat in meatball formulation for halal authentication purposes.

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