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FULL PAPER

Duplex-real time polymerase chain reaction assay for simultaneous analysis of pork and chicken in sausage products

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Abstract

The adulteration of meat-based food is common due to the price difference among raw meat materials. One of the favorite foods commonly consumed by Indonesian and Malaysian societies is chicken sausage, which can be substituted by pork to get economical profits. The objective of this study was to develop a duplex real-time PCR assay using the EvaGreen fluorescence dye for the identification of chicken and pork in sausage products. The method involved the application of chicken (*Gallus gallus*) and pork (*Sus scrofa*) specific primers which amplify the small fragments (pork 176 bp and chicken 183 bp) of the mitochondrial D-loop 22 and mt-12s rRNA genes, respectively. DNA was isolated from raw meat materials and reference sausage made from the mixtures of chicken and pork to optimize the assay. The primers used for pork were forward 5'-TCG TAT GCA AAC CAA AAC GCC -3' and reverse: 5'- ATG CAT GGG GAC TAG CAG TTA -3', while primers used for chicken were forward: 5' TGA GAA CTA CGA GCA CAA AC 3' and reverse: 5' ACA TTG TGG GAT CTT CTA GGT 3'. Gene products of chicken and pork produced two distinct melting peaks simultaneously at 76.5 and 84.5°C, respectively. The detection limit of duplex-real time PCR analysis of the reference sausage samples was 0.5% for pork and chicken meat in sausage products. The coefficient of variation (CV) of threshold cycles (Ct) for amplification was 6.25%, lower than that required by the Codex Alimentarius Commission. Duplex-real time PCR analysis followed by melting curve analysis offered rapid, sensitive, and specific detection of pork and chicken in sausage products.

1. Introduction

Chicken sausages are one of the commonly consumed meat-based products worldwide. Due to the price difference among raw meat materials, some unethical sellers try to substitute chicken with pork (Kitpipit *et al*., 2014). Since the scandal horse meat adulteration in 2013, the authentication analysis of meat-based food products is fast growing field having relevance to religious due to the restriction of certain meat such as pork for Muslim and Jew communities (Ali *et al*., 2014), health aspects such as bovine Spongiform Encephalopathies (BSEs) diseases due to bovine consumption (Salman *et al*., 2012), fair-trade policy (Bottero and Dalmasso, 2011), and cultural-related issues (Haider *et al*., 2012).

The possibility of pork is used as a mixture of sausage formulation has been the main concern for the Islamic followers, therefore, it is necessary to develop a specific and selective method of identifying pork in food products (Kesmen *et al*., 2007; Soares *et al*., 2013). Several methods have been developed for the detection of pork adulteration in food products such as meatballs and sausages, namely FTIR spectroscopy combined with multivariate data analysis (Rohman *et al*., 2011), nuclear magnetic resonance (Nurrulhidayah *et al*., 2015), the combination of electronic nose and gas chromatography-mass spectrometry (Nurjuliana *et al*., 2011), differential scanning calorimetry (Mansor *et al*., 2012), two-dimensional gas chromatography-mass spectrometry time of flight (Indrasti *et al*., 2010), and liquid chromatography (Rohman *et al*., 2012). Some of these methods are lack in sensitivity such as FTIR spectroscopy and complex instrumentation like NMR spectroscopy and 2D chromatography, therefore, specific method based on the DNA detection using real-time polymerase reaction has emerged as method of choice

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for detection of pork in food systems (Aida *et al*., 2005; Che Man *et al*., 2007).

Duplex-real time PCR analysis and other multiplex assay offered fast, sensitive, specific, and cost-effective techniques for the detection of animal species in food products based on the amplification of specific primers on DNA targets (Safdar *et al*., 2012; Motalib Hossain *et al*., 2017). This technique has been successfully used for detection of pork adulteration in bovine-based food (Soares *et al*., 2010), discrimination of DNA raw meats of bovine, buffalo and porcine in food chain for halal authentication (Hossain *et al*., 2017), identification of non-halal meats of pork, canine, rat, cat, and monkey (Ali *et al*., 2015), identification of beef, pork, horse and sheep species (Koppel *et al*., 2011), identification of beef, pork, lamb, chicken, ostrich, and horse meat (Kitpipit *et al*., 2014), and identification of DNA of seven meats namely beef, turkey, chicken, pork, horse meat, sheep and goat (Koppel *et al*., 2009). Identification of chicken DNA in a mixture of pigs, fish, and poultry in feed ingredients using chicken forward primers: 5'TGA GAA CGA GCA CAA AC 3' and reverse: 5'ACA TTG TGG GAT CTA GGT 3' has been successfully carried out by *multiplex* PCR (Dalmasso *et al*., 2004). The study used a PCR instrument, which requires the electrophoresis stage to determine the results of DNA amplification because each amplification cycle cannot be observed directly. Identification of pig DNA in a mixture of pork and chicken meatballs by real-time PCR method using primers targeting the mitochondria DNA (mtDNA) D-Loop22 has been successfully designed by Rohman *et al.* (2017). The real-time PCR method can be quantified specifically, and without requiring further stages. The real-time PCR method is done in one stage, to reduce the possibility of error or loss of sample and sample contamination during the transfer process. The method that has been developed is a testing method that identifies one type of meat in a single test (singleplex), so it has not been able to identify simultaneously which meat mixture is contained in the food sample. For this reason, it is necessary to develop a method that can identify two or more types of meat in one test without further stages that can identify more than one pair of primers, namely the multiplex real-time PCR.

However, no duplex-real time PCR assay has been reported for the differentiation of heavily consumed meats in sausage products, namely pork and chicken. Therefore, the objective of this study was to develop reliable and cost-saving duplex-real time PCR assay for identification of DNAs of pork and chicken simultaneously targeting two different genes in mitochondrial sites.

2. Materials and methods

2.1 Materials

The raw meats used in this study namely pork, chicken, beef and others are purchased from local markets around Yogyakarta, Indonesia. Preparation of chicken sausages was carried out according to Pebriana *et al.* (2017). The reference of 100% chicken sausage, 100% pork sausage and the mixture of pork-chicken sausages were prepared in the laboratory. Minced pork-chicken meat was mixed with tapioca flour, egg, and spices (garlic, pepper, salt, and sugar). The meat and other ingredients were transferred into sausage casings before being boiled in water for 15 min. The sausages were then stored at −20°C before being used for analysis.

2.2 Oligonucleotide primers

The set of primers specific for pig species was designed by Rohman *et al.* (2017), while primers designed by Dalmasso *et al.* (2004) were used for amplification of chicken DNA (Table 1).

Table 1. set of primers used for analysis of chicken DNA and pig DNA

Species	Forward primer	Reversed primer
Pig	5'- TCG TAT GCA AAC CAA AAC GCC -3'	5'- ATG CAT GGG GAC TAG CAG TTA -3'
Chicken	5' TGA GAA CTA CGA GCA CAA AC 3'	5' ACA TTG TGG GAT CTT CTA GGT 3'

2.3 DNA Isolation

The procedure of DNA isolation in sausage and fresh meat was carried out according to Sambrook *et al.* (1989). Each fresh meat as well as laboratory-made and commercial sausages were cut into small and were grounded with mortar and stamper. A-200 mg of grounded samples was added with 1000 µL of lysis buffer comprising Tris HCl, EDTA, NaCl, and SDS 1%, added with 30 µL proteinase K (20 mg/mL) and then vortexed for 5 mins. The mixture was incubated at 55^{o}C for 1 hrs, and then centrifugated at 13.000 rpm for 15 mins. In a new microtube, the supernatant was added with cold phenol (0.5 x volume), shaken for 30 mins with shaker, and followed by centrifugation at 13.000 rpm for 10 mins. The supernatant was transferred into a new micro-tube, added with chloroform (0.5 x volume), homogenized, and centrifugated at 13.000 rpm for 10 mins. The supernatant in a new micro-tube was taken, added with Na-acetate 3M pH 5.2 (0.1 x volume) and absolute ethanol (2 x volume), and incubated at -4^{o}C overnight. The pellet containing DNA was subsequently washed with 250 µL ethanol 70%, and dissolved in 50 µL TE buffer. DNA obtained was stored at -20^{o}C for further analysis.

2.4 Analysis using multiplex real-time PCR

Analysis using real-time PCR was carried out with instrument of PCR CFX96 (Bio-Rad, USA) using total volume of 10 µL, which consisted of consisting of 5 µL SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), 0.5 µL forward primer of pig 5 µM, 0.5 µL reverse primer of pig 5 µM, 0.5 µL forward primer of chicken 5 µM, 0.5 µL reverse primer of chicken 5 µM, 0.5 µL mixed DNA pork-chicken (145.65 ng/µL), and 2.5 µL nuclease free water. The thermocycler of PCR was programmed as follows: pre-denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 15 s, annealing temperature was optimized at 50-60°C for 30 s based on Tm of primer, and elongation at 72°C for 10 s.

2.5 Validation of real-time PCR

Validation of real-time PCR analysis was carried out by assessing numerous characteristics performances including the sensitivity expressed with detection limit, efficiency and precision. The validated method was subsequently used for the analysis of commercial sausage.

3. Results and discussion

Duplex-real time PCR using two primers specific to species of *Sus scrofa* (pig) and *Gallus gallus* (chicken) targeting on the mitochondrial D-loop 22 and mt-12s rRNA genes was developed for identification of chicken sausages adulterated with pork for halal authentication study. DNA was obtained from the isolation of fresh tissue of raw meats or sausage samples containing pork and chicken using chloroform-phenol-isoamyl alcohol according to Sambrook *et al.* (1989). The DNA isolates obtained were analyzed qualitatively using 0.8% agarose gel electrophoresis. The purity of DNA isolates and quantitative analysis was performed using a UV spectrophotometer at a wavelength (λ) of 260 and λ280 nm. Gel electrophoresis was used to verify the DNA integrity since the variations of DNA fragment length which used as the parameter for DNA integrity are dependent on samples analyzed, the extensive degree of food processing and the DNA extraction method.

Figure 1 reveals the DNA profiles extracted from raw materials of meat [A] and those extracted from chicken-pork sausages [B], in which the DNAs extracted from reference sausages showed intense smears of short and long DNA molecules, which indicated that DNA was not degraded excessively during extraction (Mafra *et al*., 2008). The ratio of absorbance values at 260 nm and 280 nm (A_{260}/A_{280}) was between 1.7 and 1.97 for all extracted DNAs, therefore, this extraction procedure ensured that good quality DNA was obtained from all

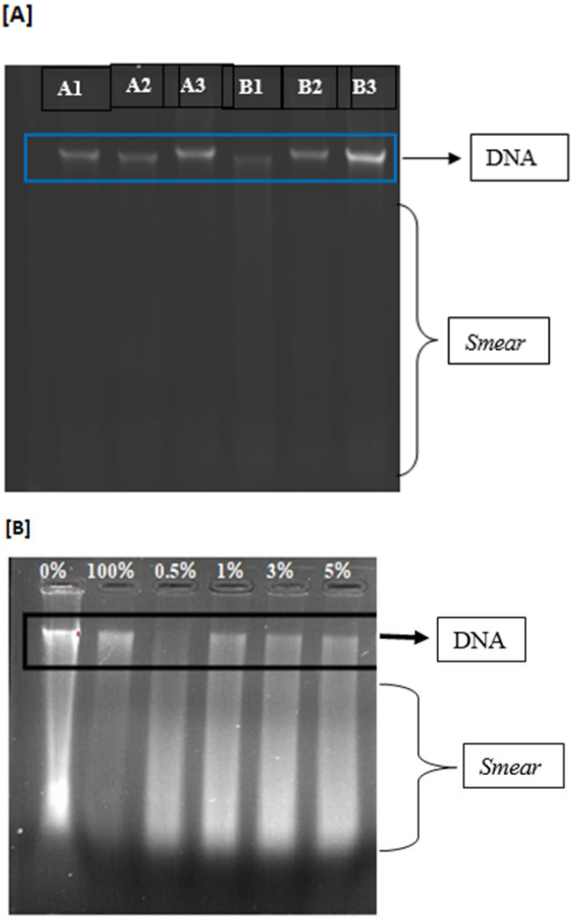

Figure 1. The agarose gel electrophoresis of DNAs extracted from raw meats of chicken (A1, A2, A3) and pork (B1, B2, B3) [A] and DNAs extracted from sausages containing 0% chicken-100% pork (0%), 100% chicken-0% pork (100%), 0.5% of pork-99.5% chicken (0.5%), 1% of pork-99% chicken (1%), 3% of pork-97% chicken (1%), and 5% of pork-95% chicken (5%) [B].

samples and it was suitable for running of PCR reaction (Nejad *et al*., 2014).

The annealing temperature is subjected to optimization at the temperature range of 50-60°C. At this temperature, DNAs extracted from pork and chicken were amplified to get the maximum response having the highest relative fluorescence unit (RFU) values with the lowest quantification cycle (Cq) value. Figure 2 shows the amplification curve for the optimization of annealing temperature using primers specific for pig and chicken having annealing temperature of 59.40°C. The amplification products of DNAs extracted from chicken and pork produced two distinct melting peaks simultaneously at 76.5 and 84.5°C, respectively. There are no dimer primers and non-specific products observed. The optimum annealing temperature of a specific primer for both pig and chicken were of 59.40°C, therefore, this temperature was used to perform duplex-real time PCR analysis. Things to consider in doing *duplex* real-time PCR mainly the temperature

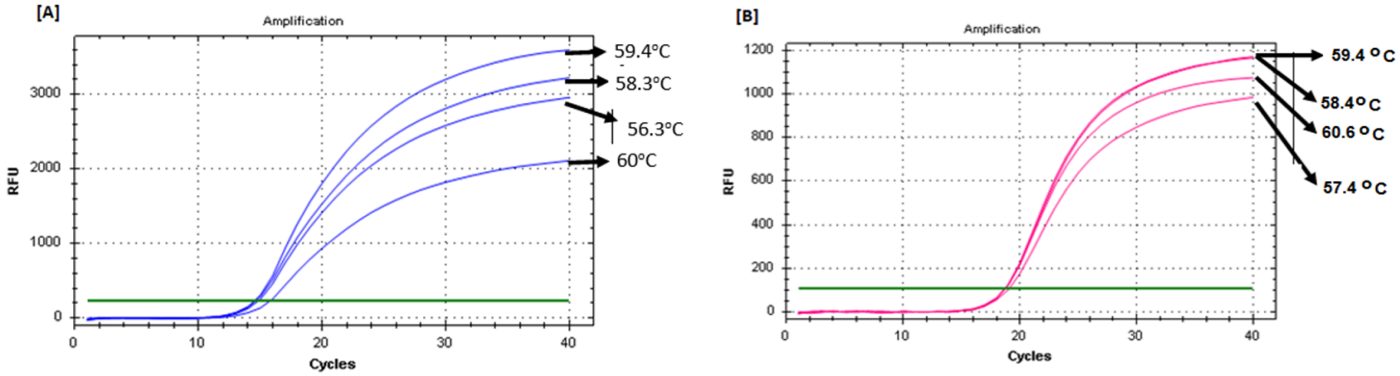

Figure 2. The amplification curve obtained during the optimization of annealing temperature using primers specific for pig [A] and chicken [B].

annealing, several pairs of primers designed must have the same annealing temperature as each other. The specificity of primers was tested for amplification of DNAs extracted from the reference sausages containing chicken and pork. The results of the duplex RT-PCR test for simultaneous detection of pig and chicken DNAs produced a single curve with two peaks having a melting temperature (Tm) value of 75.50°C for pig and 84.00°C for chicken. This Tm difference could be used as the indicator for the presence of chicken meat and pork in sausage products. Figure 3 exhibits the melting curve analysis obtained during the amplification of pork and chicken DNAs using specific primers having a melting temperature (Tm) value of 75.5°C for pig and 84.0°C for chicken.

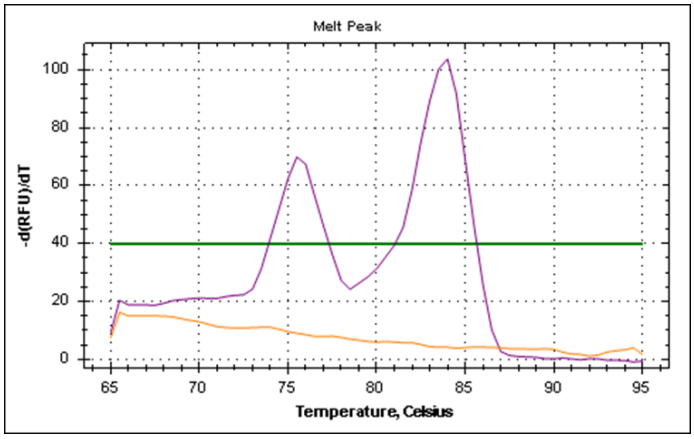

Figure 3. The melting curve analysis obtained during the amplification of pork and chicken DNAs using specific primers having a melting temperature (Tm) value of 75.50°C for pig and 84.00°C for chicken.

The validation of duplex-real time PCR was performed by assessing the parameters of sensitivity expressed by the limit of detection (LoD) of DNAs extracted from chicken and pork, the repeatability test for precision evaluation, and the efficiency value (E) of amplification. During the evaluation of LoD, DNAs extracted from the mixture of raw meats of chicken and pork as well as DNAs extracted from reference sausages were subjected to serial dilutions to get 100; 10; 1; 0,1; and 0.01 ng (raw meats) and 0.5; 1; 3; and 5% of pork. The primers could amplify DNAs of chicken meat and pork as low as of 0.01 ng corresponding to 0.5% of meats in sausage samples (Figures 4 and 5). The R^2 value obtained was 0.993 and 0,963 for reference sausages, which meet the requirements according to Codex Alimentarius commission (2010). The efficiency value obtained was 107.5% (acceptance criteria of 90 - 105%). The repeatability test showed that coefficient variation (CV) was 6.25% which meets requirements for PCR assay, namely CV ≤ 25% (Codex Allimentarius Commission, 2010). Analysis of pork DNA to chicken sausage samples did not indicate the presence of pork because there are no amplification peaks corresponding to pig DNA (Figure 6).

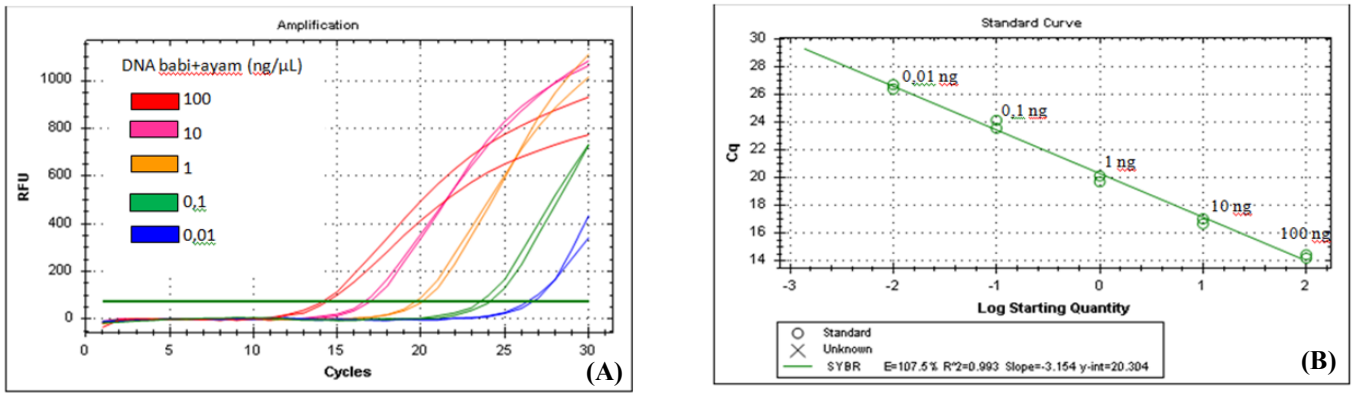

Figure 4. The sensitivity results of fresh DNA tissue dilution series of pigs: chicken with duplex-real time PCR with concentrations of pig and chicken DNAs concentration: chicken 100; 10; 1; 0.1; and 0.01 ng. (A) amplification curve; (B) linear regression for determination efficiency value.

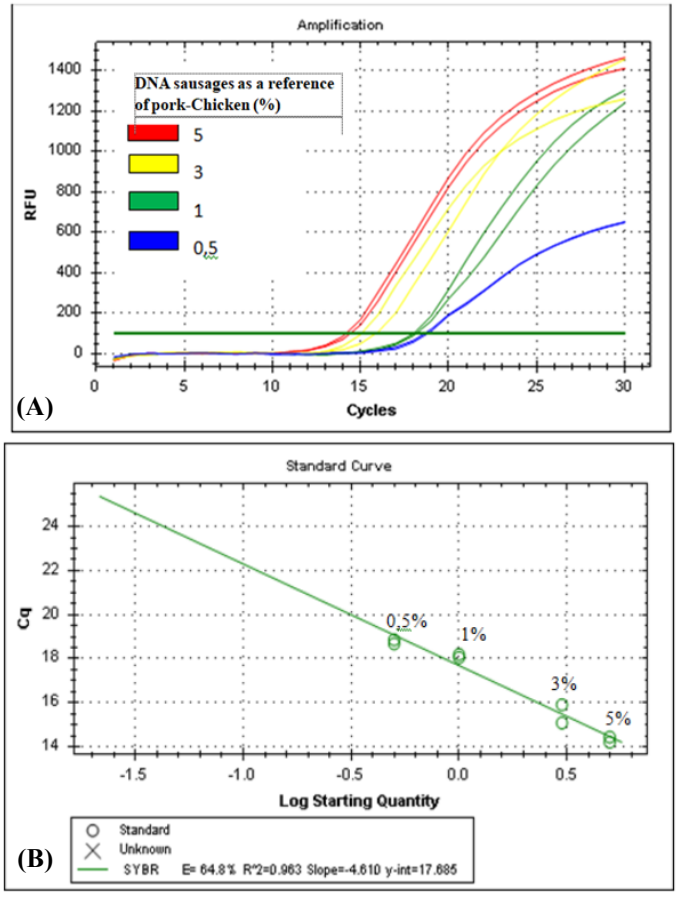

Figure 5. The sensitivity result of sausages as a reference of pork-chicken concentration 0,5; 1; 3; and 5%. (A) amplification curve (B) linear regression for determination efficiency value

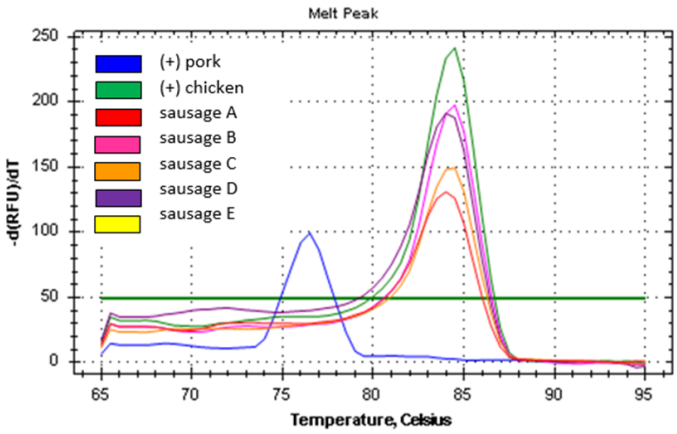

Figure 6. The amplification curves of DNA extracted from commercial samples of sausages. No amplification is found for all samples tested.

4. Conclusion

The method of duplex-real time PCR using species-specific primers can detect pork and chicken DNAs in chicken sausage products simultaneously. Detection limits on a mixture of DNA of fresh pork-chicken and pork-chickens mixed sausages were 0.01 ng and 0.5%, respectively. Duplex-real time PCR analysis followed by melting curve analysis offered rapid, sensitive, and specific detection of pork and chicken in sausage products and therefore could be proposed as standard method for meat species detection in meat-based food products for halal authentication.

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