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Detection of DNA pork in processed meat products with real-time polymerase chain reaction

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Abstract

Real-time polymerase chain reaction (qPCR) technique is a suitable method for identifying animal species in processed meat because of its ability to amplify a few fragments of DNA. A specific fragment of pork (*mitochondrial cytochrome b gene* (*cyt-b*)) was used as a DNA ladder. This study aimed to evaluate the use of a *cyt-b* gene generated primer for detecting the presence of pork in processed meat products by qPCR and determining the threshold cycle cut-off. The evaluation of the primer effectiveness was performed by threshold cycle (Ct) value, amplicon size by electrophoresis and melting curve. Two corned (A, B) and two jerkies (C, D) collected from the market were used as the sample. Genomic DNA from samples, fresh beef (as negative control) and fresh pork (as positive control) were extracted using Qiagen Kits. Amplification condition for 50 cycles of the *cyt-b* gene was performed as the initial step at 95°C for 10 mins, denaturation step at 95°C for 15 s, annealing step at 55°C for 60 s, extension step at 72°C for 30 s and post-PCR at 72°C for 3 mins. The threshold cycle (Ct) cut-off less than 30 confirmed as pork positive. The result obtained indicated that sample A and D were pork negative, with Ct value respectively 40.73 and 43.59. Melting temperatures of amplicon were ranged from 79.5 to 80.5°C, only differed by 1°C, and the amplicon electrophoresis resulting in a single band of the same size (149 bp). Hence, the melting curve analysis and electrophoresis of PCR products were not able to differentiate between pork and beef.

1. Introduction

Mixing of processed meat products such as bacon, corned beef or shredded beef with pork needs to be addressed. Substitution of meat or other parts of the pig must include a special mark of the words "contains pork and pork picture" in red text in the red box above the white base following the Regulation of the Head of Indonesian National Agency of Drug and Food Control No. HK. 03.1.23.06.10.5166 (2010). Analytical methods of detection of pork in processed meat products generally use DNA as a marker. DNA sequences can be used to identify a species. DNA is also more stable against treatment processes such as cooking and sterilization processes (Ballari and Martin 2013).

Heating raw meat at a temperature of 100 and 120°C for 30 mins can cause DNA damage, but by using *mitochondrial cytochrome b gene* (*cyt-b*) types of meat, it can still be identified (Hsieh *et al.*, 2005). Mitochondrial DNA in mammalian cells contains 37 genes coding for 22 tRNAs, 2 rRNAs and 13 mRNAs (Lee *et al.*, 2016), small (15-20 kb) and circular. Mitochondrial DNA is an efficient tool as a molecular marker compared to nuclear DNA to detect the type of meat.

One of the methods to detect meat substitution is Polymerase Chain Reaction (PCR) technique. PCR is an in vitro technique that amplified DNA segment using primer pair based on temperature changing (Kumari and Thakur, 2014). Researches on DNA detection of meat have been applied using real-time PCR (Kesmen *et al.*, 2013; Soares *et al.*, 2013; Al-Kahtani *et al.*, 2017). The advantages of using real-time PCR are the use of a closed tube thus avoiding the mix of DNA to be analyzed, speed up analysis time, and high specificity (Hedman and Rådström, 2013).

This study aimed to determine the ability of a

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commercial kit from Qiagen to isolate DNA from processed meat and to test the specificity of real-time PCR method that had been validated with dye EvaGreen™ to detect pork substitution in raw meat and processed meat based on the presence of mitochondrial *cyt-b*.

2. Materials and methods

Samples used for the DNA isolation were a positive control (pork, pork jerky, and corned pork), a negative control (beef and corned beef) and meat samples (A = corned 1, B = corned 2, C = jerky 1, D = jerky 2). For the DNA isolation we used commercial DNA isolation kit (Qiagen) consisting of AL buffer, buffer AW1, buffer AW2, AE buffer (elution buffer) and proteinase K; CTAB pH 8.0 containing 20 g/L CTAB (Sigma); ethanol absolute pro analysis (Merck). Reagents for electrophoresis were molecular grade agarose (Invitrogen), SYBR Safe 10.000x in DMSO (Invitrogen), 1x TAE buffer (40 mM Tris-Acetate, 1 mM EDTA) (1st BASE), ddH_2O, 100 bp DNA ladder (Promega), blue orange 6X loading dye (Promega). Materials for real-time PCR were SsoFastTM EvaGreen ® Supermix (Bio-Rad), TE Buffer (1st Base), DNA, *cyt-b* forward primer 5'-ATG AAA CAT TGG AGT AGT CCT ACT ATT TAC C-3', *cyt-b* reverse primer 5'-CTA CGA GGT CTG TTC CGA TAT AAG G-3'. The equipment used was an electrophoresis device, UV-1800 spectrophotometer (Shimadzu), Gel documentation system® ChemiDoc™ XRS (Biorad) and Real-time PCR IQ5™ (Biorad).

2.1 DNA Isolation

Briefly, 200 mg from 200 g sample that had been mashed was weighed in a 1.5 mL microcentrifuge tube. One mL of CTAB was added and mixed well, centrifuged at 13000 rpm for 1 min. Supernatants were removed as much as possible and left about 200 mL supernatant. After 30 µL proteinase K was added into the tube, the mixture was incubated at 65°C for 20 mins and vortexed when it finished. The mixture was incubated once more at 65°C for 20 min after had been added 300 µL buffer AL and vortexed for 10 s. Absolute ethanol (500 µL) was added to the mixture, vortexed for 10 s, and then centrifuged at 10000 rpm for 2 mins. Afterwards, the mixture was transferred to the *QIAamp* spin column and placed in a clean 2 mL collection tube and centrifuged at 8000 rpm for 1 min. The *QIAspin* column was washed twice by washing buffer. Firstly, 500 µL Buffer AW1 was added and centrifuged at 8000 rpm for 1 min. Then 500 µL Buffer AW2 was added and centrifuged twice at 13000 rpm for 3 mins and 13000 rpm for 1 min. The *QIAamp* spin column was then placed in a clean 1.5 mL microcentrifuge tube and the DNA was eluted with 80 µL RNAse free water centrifuged at 8000 rpm for 1 min. Before it was used for the amplification, the DNA isolate was stored at -20°C.

2.2 DNA Purity and concentration

The purity of DNA was determined using UV spectrophotometer at wavelengths of 260 nm and 280 nm. The purity of DNA samples was confirmed by absorbance (A260/A280) ratio, which was 1.8-2.0. DNA concentration was determined by absorbance at a wavelength of 260 nm (1 absorbance unit equal to 50 µg/ mL of double-stranded DNA).

2.3 Visualization of DNA

Agarose in 1x TAE buffer was boiled to obtain 2% w/v. After the temperature reached 70°C, SYBR Safe 10.000x in DMSO was added into agarose and then poured into the gel mold. The gel that had hardened was then placed into an electrophoresis set containing 1x TAE buffer until the gel was submerged. 10 µL of DNA was mixed homogeneously with 2 mL loading dye and loaded into the wells. 10 kb DNA ladder was included on one of the wells. Electrophoresis was conducted at 75 volts for 1 hr. The results were visualized using a gel documentation system under UV light.

2.4 Amplification of the cyt-b gene using real-time PCR

The PCR primers used to detect the *cyt-b* gene fragment in food in the PCR amplification were *cyt-b* forward primer 5'-ATG AAA CAT TGG AGT AGT CCT ACT ATT TAC C-3', *cyt-b* reverse primer 5'-CTA CGA GGT CTG TTC CGA TAT AAG G-3' (Dooley *et al.*, 2004). Double-stranded amplifications were carried out in a final volume of 25 µL, containing 12.5 µL *SsoFast TM EvaGreen® Supermix* (Bio-Rad), 0.75 µL each of forward and reverse primer (30 pmol), 2.5 µL of template DNA, and 8.5 µL ddH_2O. The PCR cycle was programmed according to the following conditions: initial activation at 95°C for 10 mins followed by 50 cycles of 95°C for 15 s, 55°C for 1 min, 72°C for 30 sec (López-Andreo *et al.*, 2005) using thermal cycler IQ5 (Bio-Rad). The specificity of PCR products was monitored by melting curve analysis and characterized by specific melting temperature (Tm) (Halliday, 2011).

2.5 Determination of samples containing pork

The decision of pork mixture in the sample was decided using the comparison of Ct values and melting temperature (T_m) of amplicon DNA bands and compared with positive controls and negative controls.

3. Results and discussion

3.1 DNA purity and concentration

The DNA quality was measured by UV spectrophotometer, the absorbance ratio at A_{260}/A_{280} nm of pork, pork jerky, beef, sample C and D was in the range of 1.8 to 2.0. The average of the values used as the accepted A_{260} quantification value for respective samples typical A_{260}/A_{280} ratios was 1.8–2.0 (Shokere *et al.*, 2009). Samples of corned pork, corned beef, sample A and B which were processed in high temperature during the canning process had a higher A_{260}/A_{280} ratio value (3.39 to 7.92).

For pure DNA, the observed 260/280 nm ratio would be near 1.8. Meanwhile, elevated ratios usually indicate the presence of RNA (corned pork, corned beef, sample A and B) as stated by Islam *et al.* (2012). UV-Vis absorbance measurements did not distinguish between DNA and RNA, so the presence of RNA would increase the absorbance at 260 and A_{260}/A_{280} ratios (Weiner *et al.*, 2007). Corned products, which had been applied to a thermal process before, could enhance the degradation of DNA especially *cyt-b* because of a strong correlation between the linear DNA degradation activity at higher temperatures (Seki *et al.*, 2008). Heat treatment could cause DNA to degrade into smaller fragments (Paunescu *et al.*, 2013). Even, the small DNA fragments would be amplified by real-time PCR. Degraded DNA would reduce its ability to bind dyes in gel electrophoresis.

3.2 Visualization of total DNA by gel electrophoresis

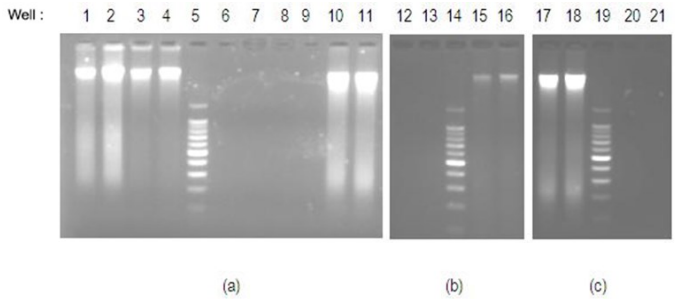

Figure 1. Total DNA electrophoresis: (a) 1 and 2 pork, 3 and 4 beef, 5 DNA ladder, 6 and 7 corned pork, 8 and 9 corned beef, 10 and 11 pig jerky; (b) 12 and 13 Sample A, 14 DNA ladder, 15 and 16 Sample D; (c) 17 and 18 Sample C, 19 DNA ladder, 20 and 21 Sample B.

DNA from the positive control and the four samples showed results in line with the results analyzed by spectrophotometer (Figure 1). A specific DNA band was not found in samples A and B as well as the corned pork and corned beef as a positive control. Beef and pork have a high concentration of DNA. This is shown by the thick DNA bands in Figure 1. A thick band was also shown in processed meat (pork jerky) and sample C. Whereas, no bands were found in corned samples either corned beef or corned pork. According to Muhammed *et al.* (2015), the amount of extracted DNA in processed meats would be reduced. In raw meat (beef, pork, and chicken), the amount of extracted DNA ranged for 24-29 µg/300 mg of material, while the processed products that had undergone autoclave ranged for 7-9 µg/300 mg of material. Therefore, no DNA bands appeared on corned beef products.

3.3 Amplification of DNA fragments (cyt-b) by real-time PCR

The optimum concentration of primers is critical in DNA amplification. Primer concentrations that were too high could lead to the formation of non-specific PCR end products such as primer dimers. While the concentrations that were too low could result in the small final product and amplification process would end before the plateau phase (Fraga *et al.*, 2008). The determination of optimum concentration used two concentrations of primer, 30 and 150 nm. Ct value of 150 nm primer was 7.53 primer and Ct value of 30 nm primer was 19.30 (Figure 2). According to Pestana *et al.* (2010), good Ct value was greater than 15. Fraga *et al.* (2008) and Pestana *et al.* (2010) suggested using the recommended final primer concentration 50-900 nM or 50-300 nM. The differences in PCR tools, PCR protocols, other materials in the mastermix, and DNA concentration affected the amplification of DNA.

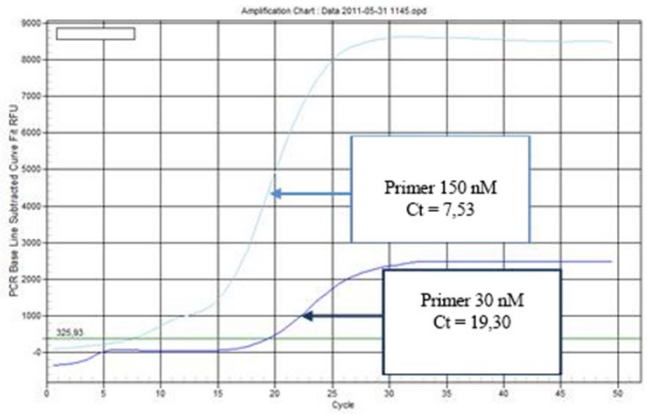

Figure 2. Ct value of two different primer concentrations

3.4 The determination of sample containing pork DNA Ct value

The content of pork can be seen from the amplification curve with Ct values. Figure 3 shows the differences in Ct values, which are 21.05 (pork) and 32.16 (beef). This is comparable to the Ct values obtained by Dooley *et al.* (2004) that were 17.41 for pork and 31.13 for beef. Specific primers should generate Ct values for the specific target and no amplification of DNA occurred in non-target species (Pestana *et al.*, 2010). The Ct values were higher than Dooley *et al.* (2004) research due to the use of different PCR dye, as

they used a *TaqMan* probe and this study used *EvaGreen*™. Additionally, the primer concentrations used were not the same, namely 175 nM by Dooley *et al.* (2004) while this study used 30 nM. The DNA concentration of corned products (16.17 ng/µL) was smaller than pork (54.60 ng/µL) and pork jerky (42.52 ng/µL). Cross-reactivity is the primer reaction of the target DNA species with non-target species. The difference in Ct values between the target species and non-target species showed cross-reactivity of 50%, double the amount of non-target species DNA would be required to give the same Ct value of the target species (Dooley *et al.*, 2004).

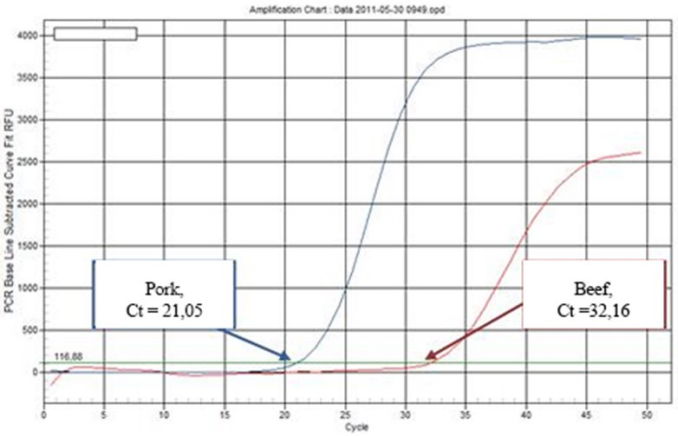

Figure 3. Amplification curve of pork and beef

This method could be used to detect the origin of species from a mixture of meat that had been processed at 121°C for 15 mins. Because the DNA target was smaller than 200 bp (149 bp), the detection of pork used a primer with the same nucleotide sequence to those used in this study. This was confirmed by Kezmen *et al.* (2009) who stated that there was no difference in Ct values found in samples of raw and roasted patty meat which produced the internal temperature 80-85°C for 5 mins. In the research of patty cooking differences (Kezmen *et al.*, 2009) the temperature was below 95°C (80-85°C) so the DNA damage could be minimized even treated in heat. It also occurred in jerky, which was treated in lower drying temperatures, so the concentration of DNA obtained was quite good.

Excessive treatment and high temperature processing in meat products will increase the degradation of DNA, because DNA can be degraded by radical and heat. This will increase the difficulty of analyzing DNA. Therefore, the Ct value of canned corned beef with high temperature (121°C, 15 mins) will be much higher than the raw meat and jerky.

The determination of sample types to a product is made by the cut-off value (Ct). Species identification was difficult to decide whether a high Ct value could be expressed as the concentration of the signal indicating low target species (pork) in a sample or the presence of cross-reactivity of species at a high level (Kezmen *et al.*, 2009). Dooley *et al.* (2004) stated that there was no cross -reactivity between the specific primers and probes for each target species and non-target species when the Ct value was 30 (cut-off value). Al-Kahtani (2017) also reported the Ct value of the binary meat mixture of beef and pork of 1, 5, 10, 20, and 100% respectively were 23.9, 24.26, 22.78, 19.09, and 16.4. In his research, Ct with specific probes were 17.41 (pork), 31.13 (cows), 37.08 (chicken), 30.00 (lamb), and 34.65 (turkey). Kezmen *et al.* (2009) found Ct value for pork was 17.69 while Li *et al.* (2019) suggested Ct value of pork was 14.92. So, the Ct values above 30 were considered negative pork and were used as a limit. The standard of Ct value for beef and corned beef respectively were 33.57 and 34.74.

Based on the average of Ct value, samples B and C with Ct values below 30 were positive as a mixture of pork. On the contrary, samples A and D which had Ct values above 30 were stated as negative samples of pork mixture. Sample A Ct value was 40.73, while sample B Ct value was 29.01. DNA concentration of sample A (16.21 ng/mL) was higher than the DNA concentration of sample B (16.08 ng/mL). Sample C DNA was amplified at low Ct and showed low Ct value that means containing a high amount of meat primer targets. Sample D Ct value was 43.59 and the concentration of DNA for sample D was 89.35 ng/mL. It was significantly higher than pork jerky (42.52 ng/mL) as a positive control.

3.5 Melting temperature (T_m)

Melting temperatures of amplicon were ranged from 79.5 to 80.5°C or differed by 1°C. Therefore, the amplicons from all samples had only one final PCR product and could not be used as a parameter of mixing of processed meats with pork despite using pork specific primers. Melting temperature (Tm) depends on the base composition of the amplicon. Amplicon that was formed from a homogeneous molecule primarily on size would give a single peak curve. If the product formed during the PCR process was not homogeneous it would form many peaks (Pestana *et al.*, 2010).

The deviation in melting temperature greater than 1° C also occurred in the use of real-time PCR for the detection of pathogen bacteria (Wilhelm *et al.*, 2000). These variations might occur due to the temperature distribution in the thermocycler which was not homogeneous. T_m as the temperature when 50% of amplicon in double-stranded configuration DNA depended on several factors: the concentration of double-stranded DNA, amplicon length, sequence of

nucleotides, and the solid composition in which DNA was suspended. Effectiveness of the melting temperature depended on the ability of the DNA extraction procedure to remove substances that inhibited the activity of DNA polymerase (Hedman *et al.*, 2013).

Melting temperature analysis should be one of the parameters to determine the contamination of the pork in processed meats. This was because different PCR final products would produce different melting temperatures (Fraga *et al.*, 2008). Kumari (2007) used real-time PCR uniplex and obtained Tm value of different 2°C between beef (76.2°C) and buffalo (78.2°C).

3.6 Gel electrophoresis DNA ribbon

The amplicon from the DNA amplification reaction in this study was observed with gel electrophoresis (Figure 4). The electrophoresis result showed that sample A, B, C, D positive control and negative control had amplicon size 149 pb or between DNA ladder 100 and 200 pb. This result was in line with the length of nucleotide from real-time PCR amplification. According to Dooley *et al.* (2004) primer pork mitochondrial *cyt-b*, forward primer with the sequence 336-366, and reverse primer with the sequence 446-460 would produce amplicon size 149 bp. The primer which was used by Dooley *et al.* (2004) had been confirmed with the data available in Gen Bank NCBI (National Center for Biotechnology Information) by using BLAST. BLAST results showed a value of 100% (query coverage) on the pork species both reverse and forward primers. Nevertheless, there was still cross-reactivity for the DNA amplification process. In *cytochrome b* of an ox (*Bos taurus*), the value obtained for the forward primers from BLAST results (query coverage) was 87%. While the reverse primer showed no similarity with ox *cytochrome b*. Basic melting temperature (Tm basic) of the forward primer was 58.9°C and 58.4°C for the reverse primer (Dooley *et al.*, 2004). The use of annealing temperature 5°C in this study could decrease the specificity during the amplification process, so whether there was a mixture of pork or not, the DNA would be amplified.

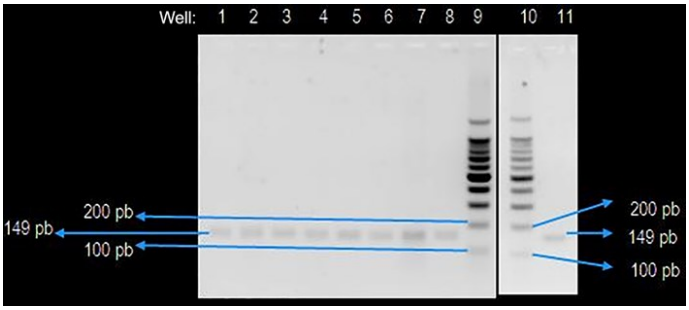

Figure 4. Electrophoresis result of real-time PCR products. (1) sample A, (2) sample C, (3) sample B, (4) sample D, (5) beef jerky, (6) corned pork, (7) pork, (8) corned beef, (9) DNA ladder, (10) DNA ladder, and (11) beef.

Thus, the melting temperatures with amplicon electrophoresis resulting in a single band of the same size (149 bp) could not be used as a parameter of meat DNA differences.

4. Conclusion

Pork detection method with real-time PCR can be an option because it is faster and relatively easy. Determination of the presence of a mixture of pork was done in three parameters that were determining the Ct values, melting temperature, and DNA electrophoresis bands. Based on the Ct value of the target DNA with cut-off value 30 on the amplification curve, samples B and C contained a mixture of pork. Whilst samples A and D which had a high Ct value and exceeded the cut-off value of 30 was declared as negative. Determination of the presence of pork DNA in meat samples using melting curve analysis and gel electrophoresis produced no difference between beef or pork or a mixture of pork. Both methods could not be used as a reference in determining the presence of a mixture of pork in this study. Further studies are needed to evaluate the DNA extraction method on samples processed meat and optimization of the annealing temperature to obtain more optimal results in PCR amplification.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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