



Research Article

Application of Primer LEP in Detecting Pork Adulteration in Meat Burger Using Hot-Start Real-Time Polymerase Chain Reaction Combined with Melting Curve Analysis

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ABSTRACT

The development of pig species detection in food is increasing due to pork adulteration. Hitherto, the strongest method to detect and quantify pig presence in food is Real-Time Polymerase Chain Reaction. The object of this study is to know whether published primers that amplified leptin gene (LEP primer) could be used to detect and quantify pig's presence in meat burger using Real-Time Polymerase Chain Reaction with dye intercalator-based detection. Genomic DNA isolation was done by proteinase K digestion. Porcine DNA was amplified using LEP primer with Hot-Start Real-Time Polymerase Chain Reaction combined with Melting Curve Analysis. Condition of Real-Time PCR used in this experiment could amplify not only 152bp porcine leptin gene fragment with T_m value of 83.5°C but also 205bp cow's leptin gene fragment with T_m 80°C . The result suggests that LEP primer is not a species-specific primer so it can't be used to quantify pig's presence in meat burger using Real-Time Polymerase Chain Reaction with dye intercalator-based detection.

Keywords : LEP primer, Real-Time PCR, pig, burger

1. Introduction

Pork adulteration in food product is common practice due to economic advantage (Fajardo *et al.*, 2010). Pork is mixed with beef in meatballs, burger, nugget, and sausage. Since pork meat consumption is forbidden in Islam, Judaism, and Advent Christian religion (Ali *et al.*, 2011), there's a need to develop a reliable, fast, and sensitive method to detect and quantify pig's presence in food product for consumer's right protection.

The fast and reliable method to detect pig's presence in food is Polymerase Chain Reaction (PCR) method. Amplification using universal primers in Restriction Fragment Length Polymorphism combined with PCR (RFLP-PCR) has been successfully used to identify pig species in food product (Erwanto *et al.*, 2009; 2011). RFLP-PCR needs additional steps before gel

electrophoresis, which is cutting PCR products with restriction enzyme. Thus, RFLP-PCR takes longer time than amplification using specific primer. Pig species detection in food product using specific primer has been demonstrated by Meyer *et al.* (1994) and Alaraidh (2008). They analyzed the PCR product using gel electrophoresis. Their results indicated that LEP primer is a pig-specific primer.

Recently, end-point PCR is being replaced with Real-Time PCR. Detection system on Real-Time PCR is carried out using fluorescence signal. This enables amplification process to be followed in real-time. Real-Time PCR is faster and more sensitive than end-point PCR because the need of using gel electrophoresis is eliminated in Real-Time PCR. Fluorescence signal in Real-Time PCR can be generated by sequence specific probe (TaqMan probe, Molecular Beacon, and Hybridisation probes) and

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dye intercalator (SYBR Green and EvaGreen). Amplification product specificity in Real-Time PCR using dye intercalator as detection system is known from Melting Curve Analysis.

The object of this study was to assess the use of LEP primer in Real-Time PCR combined with Melting Curve Analysis to detect pig's presence in mixed meat burger. It was indicated that LEP primer could amplify both cow and pig's DNA.

2. Materials and Methods

2.1. Sample preparation and DNA extraction

Raw meat samples were purchased from local market in Yogyakarta and processed into burger meat immediately. Both raw pork and beef samples were minced and blenderized individually. Meat burger consisted of 20 mg raw meat, egg, bread crumb, and salt. Pure beef burger was made as negative control and pure pork burger as positive control. Mixed meat burger was made using 50% concentration of added pork to beef burger. Burger samples were stored in -20°C . Different parts of meat burger were homogenized using mortar and stamper. A hundred milligrams of homogenized sample were transferred into 1.5 ml eppendorf tube and DNA was extracted with 1 ml lysis buffer and 10 μl proteinase K followed by incubation at 55°C overnight. After incubation, a mix of phenol, chloroform, and isoamyl alcohol (25:24:1) was added at the same volume as lysate sample. Samples were shaken for 30 minutes then centrifuged at 12,000 rpm for 5 minutes. Upper phase were transferred into new eppendorf and DNA was precipitated by sodium acetate and ethanol absolute. DNA pellet was then stored in TE buffer at -20°C .

2.2. Real-Time PCR protocol

The Real-Time PCR was carried on the CFX96 Real-Time PCR System (Bio-Rad) and analysed using CFX Manager Software (Bio-Rad). The 20 μl reaction mixtures contained SsoFast™ EvaGreen® Supermix, 500 nm LEP forward primer, 500 nm LEP reverse primer, 20 ng of DNA, and nuclease-free water. The LEP forward primer sequence is 5'-TGCAGTCTCTCCTCCAAA-3' with T_m value of $57,6^{\circ}\text{C}$. The LEP reverse primer sequence is 5'-CGATAATTGGATCACATTCTG-3' with T_m value of $56,7^{\circ}\text{C}$. The reaction mixture was amplified for 35 cycles with the condition of pre-denaturation at 95°C for 30s, denaturation at 95°C for 10s, annealing at 50, 52, 55°C for 10s, and elongation at 72°C for 10s. DNA extracted from pork meat burger were used as positive control while no-template control (NTC) and DNA extracted from beef burger as negative controls for the analysis. The NTC contained reaction mix with no template DNA. Real-Time PCR result then was assessed by gel electrophoresis to confirm specificity.

2.3. Efficiency

Five ten-fold DNA serial dilutions from 50 ng/ μl to 5pg/ μl of pig species were prepared to create the standard curve. The efficiency (E) of the Real-Time PCR

was calculated according to the formula $E = (10^{-1/\text{slope}} - 1) \times 100\%$ (Adams, 2006).

3. Result and Discussion

In this study, 152bp fragment of pork leptin gene was amplified using LEP primer for pig species detection in food product. Three different annealing temperature (50, 52, 55°C) was done simultaneously using thermal gradient CFX96 Real-Time PCR (Figure 1).

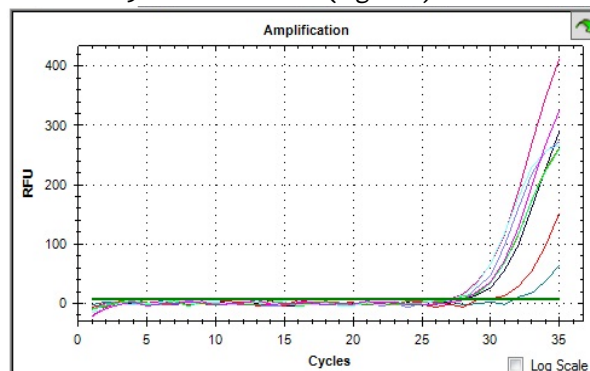


Figure 1. Amplification DNA sample at different annealing temperature simultaneously (50, 52, 55°C)
Red, was for amplification of pig's DNA template
Green, was for amplification of cow's DNA Template
Blue, was for amplification of mixed DNA template

Amplification result of Real-Time PCR was astounding. DNA extracted from beef, pork, and mixed meat burger were amplified. Melting curve analysis showed cow's amplification product has T_m value of $80,0^{\circ}\text{C}$ and pig's $83,5^{\circ}\text{C}$. Interestingly, it was showed through melting curve analysis that DNA sample from mixed meat burger had 2 peak with different T_m , $80,0$ and $83,5^{\circ}\text{C}$ (Figure 2). It was confusing because Meyer et al. (1994) and Alaraidh (2008) indicated that LEP primer was a pig-specific primer. In order for clarifying the amplification result, primer analysis using BLAST was done. BLAST result showed that LEP primer annealed at cow's leptin gene, yielding 205bp (Figure 3). Mismatch between primer and its complement base explained the phenomenon of lower amplification product at higher annealing temperature.

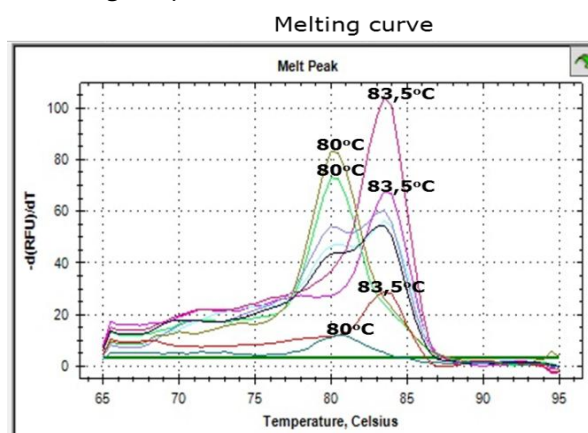


Figure 2. Melting Curve Analysis of DNA sample at different annealing temperature simultaneously (50, 52, 55°C)
Red, was for amplification of pig's DNA template
Green, was for amplification of cow's DNA template
Blue, was for amplification of mixed DNA template

It was known from pig's leptin gene data that there was LEPHINFI primers which function as STS. Base composition of LEPHINFI primers was similar with LEP primers. However, LEP forward primer lacked 3 bases and reverse primer 1 base compared to LEPHINFI primers (Figure 4). Thus LEP primers wouldn't attach perfectly with leptin gene. This explained phenomenon of lower amplification product at annealing temperature 55°C

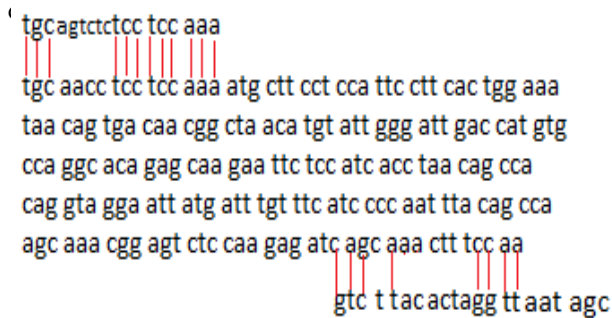
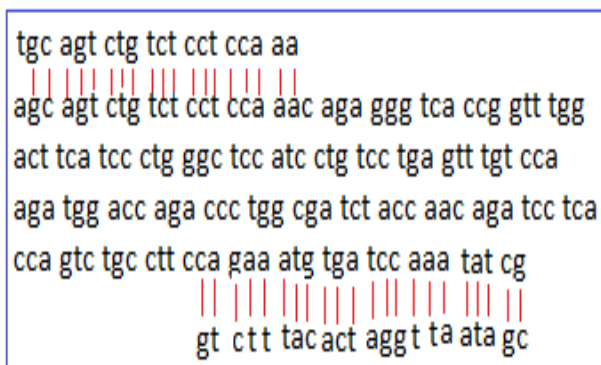


Figure 3. Annealing site LEP primer at Cow's leptin gene (a)



(b)

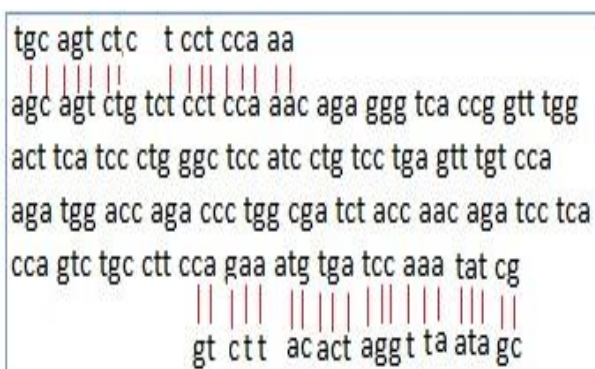


Figure 4. Annealing site LEPHINFI and LEP primer at Pig's leptin gene
(a) Annealing site LEPHINFI primer at Pig's leptin gene
(b) Annealing site LEP primer at Pig's leptin gene

Amplification product from cow's and pork's DNA was different. Cow's amplification product was 205bp with 34.80%GC and Tm 80.0°C while Pig's was 152bp with 52.63%GC and Tm 83.5°C. Pig's amplification product has higher melting temperature than cow's although its size was lower because its GC percentage

was higher. The pig's amplification product was at the same size as described by Alaraidh (2008). However, there was unreported band at 200bp from gel electrophoresis of PCR results for pig species detection in beef sausage (lane 4) and beef steak (lane 7) using the same primer. Alaraidh (2008) did not explained this 200bp band presence. Moreover, there were several bands from gel electrophoresis of PCR results for pig species detection in Turkey sausage even though the size was not the same as pig amplification product. This finding showed that LEP primer was not species-specific primer and therefore could not be used in Real-Time PCR protocol using dye intercalator (SYBR Green, EvaGreen) as detection system. Dye intercalator would intercalate with all amplification products so the generated fluorescence signal came from both cow's and pig's amplification product. Thus, the Ct value could not be used in sensitivity calculation assay rendered the use of Real-Time PCR was meaningless. LEP primer could be used in Real-Time PCR method if sequence-specific probe (TaqMan probe, Molecular Beacon, and Hybridization probe) was used as detection system. The probe could be designed to hybridize only at pig's amplification product. Thus, fluorescence signal is generated only from pig's amplification product and Ct value could be used in sensitivity calculation assay.

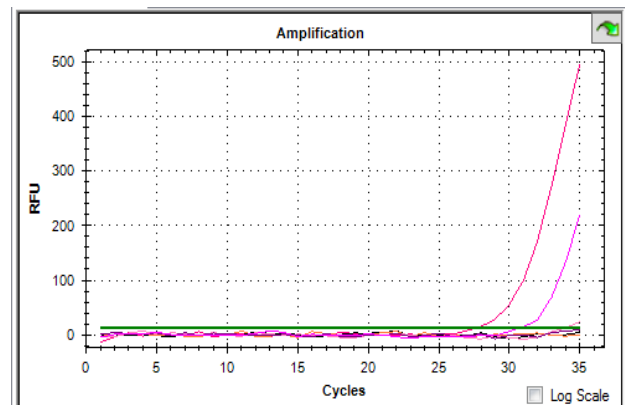


Figure 5. Amplification of standard curve

The efficiency of Real-Time PCR is a significant criterion to assess the suitability of the used protocol. Unfortunately, efficiency assessment for this procedure could not be done because only 2 out of 5 ten-fold serial dilutions were amplified (Figure 5). Amplification occurred when 50 and 5ng pig's DNA were used as template while no amplification detected at 500, 50, and 5pg. This phenomenon confirms the disadvantage of using a portion of genomic DNA which is present in 1 copy per cell (Ballin et al, 2009).

4. Conclusion

We suggest that LEP primers is not species-specific primer and can not be used in Real-Time PCR protocol using dye intercalator as detection system.

5. Acknowledgement

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