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Analysis of pork meat using loop mediated isothermal amplification (LAMP) to confirm halal status

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Abstract

A method has been developed for identifying species from pork samples by loop-mediated isothermal amplification (LAMP) analysis of the wild boar (*Sus scrofa*) mitochondrial DNA D-loop region. DNA was extracted from raw and cooked pork and beef meat and subjected to polymerase chain reaction (PCR) and LAMP. Approximately 185 base-pair PCR products were found in the pork meat samples after amplification of the D-loop region. LAMP products only showed a smear band with the pork samples. Moreover, by adding CuSO₄ to the LAMP product, the white Cu(OH)₂ precipitate, which formed a ring-shaped deposit, was not seen in the pork samples, but was seen in the beef and negative control samples. Our finding showed the LAMP technique to be a powerful molecular-biological method for species identification. The addition of CuSO₄ to the LAMP product is an alternative method that can be used in field studies, and does not require any gel-electrophoresis apparatus.

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Introduction

The identification of meat species and the authentication of its halal status are major concerns in Asia, France, Germany, Greece, Italy, Russia, Switzerland, the United Kingdom, North and South America, and many other countries. Nowadays, the food chain has become so long and people's lifestyle have changed. This has resulted in the need to preserve and process meat into various meat products (Vandendriessche, 2008). With technology advances in the meat -processing industry, adulteration and fraud have become common due to monetary benefits of substituting cheaper and more widely available produce.

Most publications have focused on the use of PCR for detecting the adulteration of meat, in an ongoing struggle against deliberate substitution of one meat species with another cheaper one. Genomic DNA from pork sausages was extracted and subjected to PCR amplification targeting pork DNA (Aida *et al.*, 2005). A PCR-RFLP (PCR- restriction fragment length polymorphism) method for pork, beef, buffalo, chicken, goat, quail and rabbit meat identification based on analysis of mitochondrial cytochrome *b* gene (cyt *b* gene) has been reported (Murugaiah *et al.*, 2009). In another report (Ulca *et al.*, 2013), the researchers used PCR analysis with traditional Turkish foods raw and cooked.

Both pork genomic DNA and mitochondrial DNA are used to detection. However, mitochondrial DNA of most species, which are circular and largely free of bound proteins contributes to greatly stability over time as it is less susceptible to degradation compare to genomic DNA (Bogenhagen, 2009). Mitochondrial DNA also survives better in highly processed foods that undergo extreme conditions during processing. In addition, the approximately of 2-10 copies of mitochondrial DNA are resulting in very high copy numbers of mitochondrial DNA per cell (Bogenhagen, 2009). So many genes in mitochondrial DNA are used for PCR amplification in pork identification. The most common target gene for species-specific PCR amplification is mitochondria-encoded cytochrome *b*

gene (Matsunaga *et al.*, 1999). The 12S rRNA and 16S rRNA mitochondria-encoded genes have also been used to identify specific species (Bottero *et al.*, 2003; Dalmasso *et al.*, 2004). At the present time, D-loop region of mitochondrial DNA is frequently chosen for meat speciation (Bellis *et al.*, 2003; Fajardo *et al.*, 2007; Fajardo *et al.*, 2008; Karabasanavar *et al.*, 2014; Rojas *et al.*, 2010).

LAMP has recently become an interesting method to replace PCR, since it is faster and more sensitive. Furthermore, LAMP has been developed and used for detection application such as detection of *Yersinia enterocolitica* in pork meat (Gao *et al.*, 2009), *Penaeus monodon* Nucleopolyhedrovirus (PemoNPV) (Nimitphak *et al.*, 2010), Human Herpesvirus (Yoshikawa *et al.*, 2004), and *Babesia gibsoni* parasites (Ikadai *et al.*, 2004). This method involved the amplification of DNA targets under isothermal conditions in the temperature range 60°C-65°C for 60 minutes (Notomi *et al.*, 2000). Two sets of primer, inner primer and outer primer sets used in LAMP were specific at six different areas located within the target sequence and primary DNA amplification started with the inner primer set. The characteristic intermediary DNA structure formed by LAMP, called a stem-loop DNA fragment, was generated and large amounts of DNA products were produced by an auto-cycle reaction (Zhang *et al.*, 2009).

In this study, the D-loop region of mitochondrial DNA was chosen as the target of this investigation. The objective of this study was to determine pork meat in Halal food by using LAMP technique. A major advantage of our method is more faster and less expensive method than PCR for identification of pork meat and another advantage is usage this technique in filed study.

Materials and methods

Collection of meat samples

Raw pork and beef samples were collected from local markets. The cooked meat samples were either cooked at 80°C with a holding period of 30 min, or autoclaved at 121°C temperature, 15-psi pressure for

20 min duration.

DNA template preparation

Genomic DNA was extracted from raw and cooked meat samples by Thermo Scientific Genomic DNA Purification Kit. The 50 mg of each raw and cooked meat samples were ground in 1.5 ml microcentrifuge tube and suspended in 200 µl of TE buffer and added 400 µl of lysis solution (in kit) and incubated at 65 °C for 5 min. After adding 600 µl of chloroform (Sigma) with gently mixing, the reaction was centrifuged at 10,000 g for 2 min. Then, the upper solution was drawn off and rawly prepared precipitate solution (in kit) was added. The solution was centrifuged at 10,000 g for 2 min and the supernatant was discarded. The remaining DNA pellet was dissolved with 100 µl NaCl solution (in kit). After that, 300 µl of cold absolute ethanol (Sigma) was added and stored at -20°C for 10 min. The solution was then centrifuged at 10,000 g for 10 min and the supernatant was discarded. The remaining DNA pellet was washed with 70% ethanol and centrifuged at 10,000 g for 10 min. The pellet was then air dried and suspended with 20 µl of sterile distilled water.

PCR and LAMP primer design

The design of the LAMP primers was based on a *Sus scrofa* mitochondrial D-loop (GenBank accession No. AF276923). The PrimerExplorer V4 software available on the Eiken Chemical Co.Ltd., website (<http://primerexplorer.jp/e/>) was used to design F3, B3, FIP and BIP primers, as shown in Table 1. F3 and B3 were also acted as PCR primer.

PCR and LAMP reaction and analysis

In PCR reaction, the amplification of the D-loop was performed in a final volume of 25 µl containing 10 ng of each extracted DNA, 1xTaq buffer (50 mM KCl, 10 mM Tris-HCl), 1.5 mM MgCl₂, 1.2 mM dNTPs, 0.8 µM of each F3 and B3 primer, and 1.25 unit of Taq DNA polymerase (New England Biolabs). PCR was carried out in a BIO RAD MJ Mini Personal Thermal Cycler. The cycle condition included a single initial denaturation at 94 °C for 3 min followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec,

and a final extension step at 72°C for 5 min. Negative controls (water) were included in each PCR amplification, in order to verify the PCR efficiency and to detect contamination.

In LAMP amplification, All reactions were carried out in 25 µl of 1xBst DNA polymerase buffer containing 5 mM MgSO₄, 400 mM betaine, 1.2 mM dNTPs, 0.8 µM F3 and B3 primers, 2 µM FIP and BIP primers, and 8 U Bst DNA polymerase (New England Biolabs), and 10 ng of each DNA extracts as a template. Reactions were incubated at 65°C for 45 min and followed by enzyme inactivated at 80°C for 5 min.

After amplification, PCR and LAMP products were analyzed by loading 10 µl of reaction products on 1.5% agarose gel. After gel electrophoresis, the gel was stained with ethidium bromide and visualized under an ultraviolet light. Only in LAMP products, 100 mM CuSO₄ was subjected to the LAMP products for the deposition of a white ring shaped precipitate.

Results

After PCR detection of *Sus scrofa* mitochondrial D-loop, the 185 base pairs band of PCR product indicates the pork meat samples. Our result revealed the positive PCR reaction was highly specific with both raw and cooked pork meat samples showed the PCR product band as shown in lane 1 and 2 in Fig. 1 while no amplicon was found in beef meat samples, as shown in lane 3, 4 and negative control in lane 5 in Fig.1.

As same as PCR amplification, LAMP reaction of *Sus scrofa* mitochondrial D-loop was specific only in pork meat samples that shown in Fig.2. After detection of LAMP product by gel electrophoresis, the smear bands of amplified products indicates the positive LAMP reaction. They showed the smear bands in raw and cooked pork meat samples in land 1 and 2 in Fig. 2 while no LAMP amplification product in beef meat samples both raw and cooked in lane 3, 4 and in negative control in lane 5 in Fig.2. In the same time, when CuSO₄ was added in each LAMP products. The white precipitate of Cu(OH)₂ that formed a ring-

shaped deposit in only negative LAMP reactions were shown in sample 3,4 and 5 in Fig. 3. While as, there was clear solution in raw and cooked pork meat sample in sample 1 and 2 in Fig. 3. So these results

indicated that the LAMP technique can be used for species identification and the addition of CuSO_4 in LAMP reaction is the easy method for directly observed the result of LAMP reaction by naked eye.

Table 1. Primer sequences used for PCR and LAMP amplification.

Primer	Sequence (5'-3')
F3	TCAACATGCGTATCACCA
B3	AGTGGGCGATTTTAGGTG
FIP	GAAGAGGGATCCCTGCCAAGATTAGATCACGAGCTTAATTACCA
BIP	CTCCGGGCCCATAAATCGTGAAGAACCAGATGCCTGTT

Discussion and conclusion

Even though, the main molecular biology technique used to detect fraud in meat products is PCR such as conventional PCR (Ahmad Nizar *et al.*, 2013; Aida *et al.*, 2005), PCR-RFLP (Murugaiah *et al.*, 2009), Real time PCR (Kesmen *et al.*, 2009), and EvaGreen multiplex real-time polymerase chain reaction (EMRT-PCR) (Safdar *et al.*, 2013). However, PCR requires sophisticated equipment and is time consuming. Therefore, a novel LAMP technique has been developed to identify meat, which was initiated by Notomi and colleagues (Notomi *et al.*, 2000). LAMP does not require and reagents or sophisticate equipment; is easy, fast, specific and sensitive, involving 4 primers base on 6 specific sequence on the target gene to generate the smear and/or ladder band. Unlike PCR, the LAMP can efficiently amplify target DNA in a short time, at a wide range of reaction temperatures, and does not require the denaturation temperature for template DNA. In addition, the LAMP had a significant advantage; the amplified product can be observed directly with the naked eye because of the precipitation of magnesium pyrophosphate ($\text{Mg}_2\text{P}_2\text{O}_7$) in positive LAMP product (Notomi *et al.*, 2000) and as fluorescence in the presence of either ethidium bromide or PicoGreen during UV illumination. This study describes that LAMP of *Sus scrofa* mitochondrial D-loop is specific only in pork meat samples.

Adding CuSO_4 to negative samples can confirm that they are indeed negative samples, so reducing the total cost of a LAMP reaction (Zoheir *et al.*, 2010).

When a positive reaction occurred, high level of pyrophosphate ions were released during amplification, which produced a visible white precipitate of magnesium pyrophosphate. When CuSO_4 was added to a negative sample, the dNTPs remained almost uncharged; they combined with the CuSO_4 to form white precipitate of $\text{Cu}(\text{OH})_2$ that formed a ring-shaped deposit. The occurrence of the latter reaction can be partly attributed to the concentration of dNTPs used in LAMP being much higher than used in PCR (Notomi *et al.*, 2000). Now, using dNTPs co-deposition, the observation of a ring-shaped deposition has been successfully used for rapid LAMP-based sexing of bovine embryos (Zoheir *et al.*, 2010) and *Columbidae* birds (Chan *et al.*, 2012). Although increasing amounts of dNTPs promoted the amount of LAMP product produced, an early LAMP step may be strongly inhibited by high concentration of dNTPs, e.g., 1.4 mM (Yeh *et al.*, 2005).

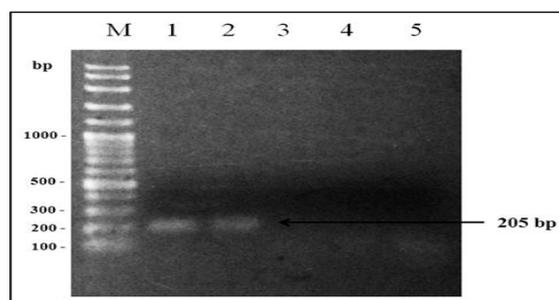


Fig. 1. Agarose gel electrophoresis of PCR products of *Sus scrofa* mitochondrial D-loop. Lane M is 100 base pairs DNA ladder. Lane 1 is raw pork meat sample. Lane 2 is cooked pork meat sample. Lane 3 is raw beef meat sample. Lane 4 is cooked beef meat sample. Lane 5 is negative control.

The accurate identification of meat products species, to detect adulteration or fraudulent substitution, and to authentication Halal, should all benefit from this new LAMP technique. This method can be conducted quickly and without complex instrumentation. It is easy to detect the results, making it suitable for field use, where gel electrophoresis apparatus is normally impractical.

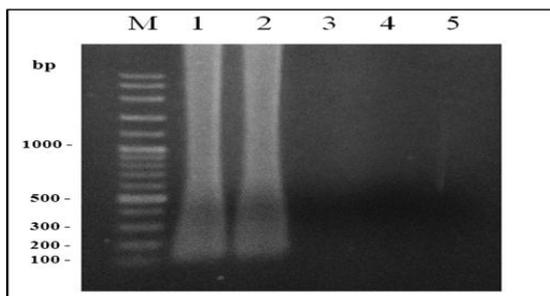


Fig. 2. Agarose gel electrophoresis of LAMP products of *Sus scrofa* mitochondrial D-loop. Lane M is 100 base pairs DNA ladder. Lane 1 is raw pork meat sample. Lane 2 is cooked pork meat sample. Lane 3 is raw beef meat sample. Lane 4 is cooked beef meat sample. Lane 5 is negative control.

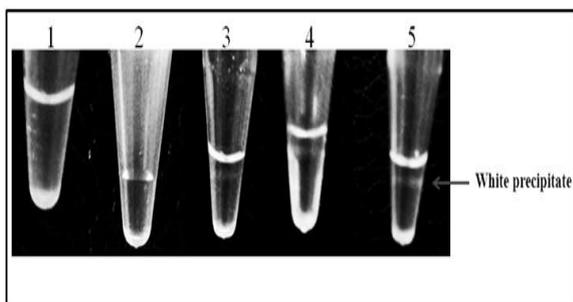


Fig. 3. White precipitation of $\text{Cu}(\text{OH})_2$ in LAMP products of *Sus scrofa* mitochondrial D-loop. Sample 1 is raw pork meat sample. Sample 2 is cooked pork meat sample. Sample 3 is raw beef meat sample. Sample 4 is cooked beef meat sample. Sample 5 is negative control.

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