Food Control 66 (2016) 53–61

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Development of a rapid on-site detection method for pork in processed meat products using real-time loop-mediated isothermal amplification

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ARTICLE INFO

Article history:
Received 10 October 2015
Received in revised form
23 January 2016
Accepted 25 January 2016
Available online 28 January 2016

Keywords:
Direct real-time LAMP
Meat authentication
Pork
On-site detection

ABSTRACT

On-site detection with minimal equipment and no risk of contamination of samples is crucial for the rapid and sensitive identification of pork in processed meat products. To resolve this issue, a reliable loop-mediated isothermal amplification (LAMP) method was developed to detect pork in meat using a portable real-time fluorometer without the need for DNA extraction. Pork-specific primers for the LAMP assay were designed based on the mitochondrial D-loop regions, and eukaryotic primers based on the 18S rRNA gene were used for the endogenous control. The adoption of an endogenous control prevented false-negative results. The detection level was 1 pg for raw pork DNA and 0.1% of pork in a beef-meat mixture. The applicability of the developed method was demonstrated in commercially processed meat products. Forty-two meat products were successfully verified for labeling compliance using this method within 30 min without the need for nucleic acid extraction.

1. Introduction

People consume meat products to obtain animal protein and have a right to be informed about the ingredients in the food they ingest. However, for economic profit, food manufacturers might replace expensive meats with cheap ones during manufacturing and use more readily available materials. This process results in meat adulteration and mislabeling of processed meat products. In the past, problems have arisen when undeclared horse meat was detected in a food product labeled as beef (Di Giuseppe, Giarretta, Lippert, Severino, & Di Maro, 2015). Pork DNA has also been identified in a number of Halal-certified products in the United Kingdom (Karabasanavar, Singh, Kumar, & Shebannavar, 2014). To address these issues, labeling controls and identification of meat species in processed meat products are considered important and urgent issues.

Pork is used as an alternative for beef or lamb in meat products because of their similar colors and textures (Wissiack, De La Calle, Bordin, & Rodriguez, 2003). The presence of pork meat in processed meat products is a serious concern; some consumers do not consume pork meat for religious reasons (Ali, Hashim, Dhahi, et al., 2012; Ali, Hashim, Mustafa, & Man, 2012). In addition, since the ingestion of pork can cause food allergies, food products containing pork should clearly display that fact on the label (Ayuso et al., 1999; Mamikoglu, 2005).

For these reasons, the methods used to detect pork have been widely studied. Reported analytical methods include anatomical, historical, microscopic, organoleptic, chemical, electrophoretic, chromatographic, and immunological assays (Barakat, El-Garhy, & Moustafa, 2014). These methods were also evaluated in processed food system and some assays were effective in detection of pork in processed meat products. For example, a monoclonal antibody-based sandwich enzyme-linked immunosorbent assay was developed for the sensitive detection of pork in both raw and heat-processed meat and feed products by Liu, Chen, Dorsey, and Hsieh (2006). They reported that heat treatment of meat samples up to 132 °C for 2 h did not affect the assay performance.

Numerous DNA-based identification methods were developed for detection of pork in processed meat products. DNA is detectable after the application of heat and pressure used in meat processing and some protein-based methods or immunological analyses have been replaced by DNA-based methods (Soares, Amaral, Oliveira, & Mafra, 2013). However, application of DNA-based method is limited in processed meat products due to the low efficiency unless

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http://dx.doi.org/10.1016/j.foodcont.2016.01.041
0956-7135/

the size of PCR amplification product is smaller than 250 bp. The effect of size of PCR amplification product on the detection efficiency was examined by researchers and DNA-based methods with small PCR amplification product were successfully applied to detect pork in processed meat products (Goldstein & Desalle, 2002; Hajibabaei et al., 2006; Sarri et al., 2014). In addition, DNA-based identification is not dependent on the tissue and can be used to analyze sequences (Fumière, Dubois, Baeten, von Holst, & Berben, 2006; Hird, Goodier, & Hill, 2003) eventhough the detection efficiency varies depending on tissue types (Iwobi et al., 2015). For this reasons, DNA-based molecular diagnostic techniques are proven to be effective for identification of pork in heat- and pressure-treated processed meat products that possibly contain various tissues such as fat, blood, gelatin, and collagen as ingredients depending on the product types.

Several DNA-based assays, such as species-specific polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) finger printing, and real-time PCR, have been developed (Calvo, Zaragoza, & Osta, 2001; Haider, Nabulsi, & Al-Safadi, 2012; Jonker, Tilburg, Hägele, & De Boer, 2008; Karabasanavar, Singh, Kumar, & Shebannavar, 2013; Li et al., 2015; Soares et al., 2013). However, the PCR-RFLP method presents ambiguous band patterns, is difficult to accurately interpret, and has a relatively long processing time. RAPD requires strict amplification conditions and therefore has low repeatability (Karabasanavar et al., 2014). Species-specific PCR and real-time PCR are more sensitive and specific than PCR-RFLP and RAPD but are time-consuming and expensive. These two methods are also not suitable for on-site detection in the field of food control.

The loop-mediated isothermal amplification (LAMP) assay is relatively fast because the method amplifies DNA in isothermal conditions. It does not require temperature changes for denaturation of the DNA, primer annealing, or extension procedures. Thus, DNA polymerase with strand displacement activity is required. Also, since this assay involves a set of four or six designed primers, including outer, inner, and loop primers, it has high sensitivity and specificity (Notomi et al., 2000). The LAMP assay is typically used for diagnosis of pathogenic bacteria and viruses, detection of genetically-modified organisms, and authentication of medicinal materials (Huang et al., 2014; Kiddle et al., 2012; Li et al., 2013; Mahony et al., 2013; Wang et al., 2015; Zeng et al., 2014; Zhang et al., 2013). A few studies have focused on the identification of animal species using the LAMP assay (Abdulmawjood et al., 2014; Ahmed, Hasan, Mosharraf Hossain, Saito, & Tamiya, 2010; Kanchanaphum, Maneenin, & Chaiyana, 2014; Ran et al., 2015; Yang et al., 2014). However, previous studies required a separate process of DNA extraction, which has limitations for field applications.

In this study, we developed a direct real-time LAMP assay to detect pork DNA in processed meat products using a portable, real-time fluorescence detector (Genie II, Optigene, UK) without the need for extraction of DNA. An endogenous control was also introduced in order to avoid false-negative results.

2. Materials and methods

2.1. Sample preparation

Raw muscle samples were obtained from the National Institute of Animal Science (NIAS), and frozen muscle tissue of some species were procured from the Conservation Genome Resource Bank (CGRB) in Korea.

To prepare binary reference mixtures, raw pork, lamb, chicken, and beef were lyophilized for 24 h using a freeze dryer (Ilsin Biobase, Dongduchon, Korea). The lyophilized samples were ground for use in the experiments. The binary mixtures of 200 mg of beef, lamb, and chicken meats were prepared with 10%, 1%, and 0.1% (w/w) of pork meat, respectively. The samples were immediately processed to isolate DNA.

Forty-two commercially processed meat products (jerky, pressed ham, sausage, hamburger patty and steak, minced rib, nuggets, cutlet, pork floss, and meatball) were purchased from retail stores in South Korea. The commercial processed meat products were selected that cover all types of processed meat products according to the Food Code in Korea (www.mfds.go.kr/index.do). The commercial samples cover highly processed meats (temperature, pressure), such as canned ham and frank sausage, and meat products contain large amounts of seasonings and food additives. The samples were stored at −20 °C until analysis.

2.2. DNA isolation

Each DNA sample was extracted from the raw meats and processed foods using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with slight modifications. Briefly, 25 mg of raw and processed meat products were lysed, and all buffers for lysis and binding to the column were used at twice the quantity specified in the manufacturer's protocol. The purity and concentration of the isolated DNA were measured using an ultraviolet spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan); DNA samples with a 260/280 nm ratio between 1.7 and 2.0 were used as template DNA.

2.3. Design of LAMP primers

DNA sequences of the mitochondrial D-loop genes of pork and eukaryotic 18S rRNA were retrieved from the GenBank database. Multiple sequences were aligned using an online multalin interface calculator (http://multalin.toulouse.inra.fr/multalin/multalin.html), and two sets of LAMP primers were designed based on the conserved regions identified using Primer Explorer software, version 4 (Eiken Chemical Co., Tokyo, Japan). Specific primers for pork were used with four other types of primers, including outer primers (F3 and B3) and inner primers (FIP and BIP). For the endogenous control, a set of four primers (two outer primers and two inner primers) was selected to perform the LAMP assay (Table 1).

2.4. Loop-mediated isothermal amplification (LAMP) reaction

The LAMP reaction was performed at a total volume of 25 μl. The reaction mixture contained 15 μl Isothermal Master Mix ISO-001 (Optigene, West Sussex, UK), 0.2 μM of each outer primer (F3 and B3), and 1.6 μM of each inner primer (FIP and BIP). Two sets of primers for pork and eukaryotes were reacted under the same conditions. The total amount of template DNA was 10 ng. The LAMP assay was performed at 65 °C for 30 min and was followed by annealing curve analysis (98–80 °C, 0.05 °C/s) to confirm the annealing temperature of each amplified sample of DNA using a Genie II LAMP detector (Optigene, West Sussex, UK).

2.5. Sensitivity and specificity

The sensitivity of a pork-specific LAMP assay was assessed using serially diluted pork DNA and binary mixtures. Pork DNA was diluted from 10 ng to 0.1 pg per reaction, and each reference mixture was used to define the limit of detection (LOD) as the lowest level of pork that could be identified. The specificity of the primers used for pork detection was tested using DNA from 18 different animal and 4 plant species (pork, *Sus scrofa*; beef, *Bos*