

Simultaneous detection of pork and wild boar meat in chicken sausages using the combination of a single primer and real-time polymerase chain reaction (qPCR)

Nurull Hikmah¹, Rumiya¹, Sisindari¹, Abdul Rohman*^{1,2}

¹Department of Pharmaceutical Chemistry, Gadjah Mada University, Yogyakarta

²Institute of Halal Industry and Systems (IHIS), Gadjah Mada University, Yogyakarta
Sekip Utara, Yogyakarta, Indonesia

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ABSTRACT

The identification of meat species in food products and pharmaceuticals is vital to minimize food adulteration practices. Due to its specificity, polymerase chain reaction (PCR)-based methods are the most commonly reported methods for detection of food adulterants. This study was aimed to evaluate the use of real-time PCR with a species-specific primer to identify two non-halal types of meat, namely pork and wild boar meat (WBM), in chicken sausages. The primer was designed using online software PrimerQuest from NCBI (National Center for Biotechnology Information) to target the mitochondrial ND1 gene of *Sus scrofa domestica*. The annealing temperature (Ta) of the primer used during real-time PCR analysis was optimized to achieve the highest response fluorescence unit at the lowest cycles. Real-time PCR using the primers of NK-ND1-Ssc1 (Forward: 5' AAAGGACCCAACGTTGTAGG 3' and Reverse: 5' TAGTGCTAGGGATAAGGCTAGG 3') was validated with several parameters, namely specificity, the limit of detection for sensitivity, linearity, efficiency, and repeatability. The optimum annealing temperature of NK-ND1-Ssc1 was 58.1°C. The sensitivity evaluation revealed that the limits of detection (LoD) of pork and WBM in reference sausage samples containing 100% pork and WBM were 500 pg and 50 pg, respectively, which correspond to 0.3% meat in sausage products. The efficiency values of real-time PCR amplification were 93.1% and 94.8% for pork and WBM with coefficient variations of 0.2884% and 0.4998%, respectively. The validated method was subsequently applied to analyze the commercial samples, and among the twelve (12) samples evaluated, there was one sample positive of containing non-halal meat (pork or WBM). Real-time PCR using species-specific primers, e.g., NK-ND1-Ssc1, is specific and sensitive; therefore, this method can be used as an alternative technique for authentication of halal meat.

Keywords: real-time PCR, non-halal meat, halal authentication, species-specific primer

*Corresponding author:

Abdul Rohman

Department of Pharmaceutical Chemistry, Institute of Halal Industry and Systems (IHIS)

Gadjah Mada University, Sekip Utara, Yogyakarta, Indonesia

Email: abdulrohmanugm@gmail.com

INTRODUCTION

Muslims currently constitute a quarter of the world population, and this share is predicted to increase (Hackett, 2015). In response to this, the halal markets in the halal industry are growing exponentially and are estimated to reach more than US\$ 1.6 trillion (Lever and Miele, 2012). The authentication of meat and meat-based food products, especially related to halal and non-halal meats, is an emerging issue in Islamic countries. Halal meat authentication has become a serious matter of grave concern for not only consumers but also food producers and meat stakeholders (Pang *et al.*, 2017; Lubis *et al.*, 2016). The common cases of meat adulteration disclosed in recent years are the substitution of pork and wild boar meat (WBM), which are non-halal, for halal meat such as beef.

The authentication of halal meat-based food requires accurate, reliable, and validated methods to unambiguously confirm the animal species in a variety of degraded and processed food matrices, such as sausages and meatballs. When available, standard methods can protect both producers and consumers from any adulteration practices and prevent illegal trafficking or overexploitation of animal species (Teletchea *et al.*, 2005). There have been numerous publications reporting developed and validated methods for confirmation and identification of meat species in food products. They typically rely upon lipid-based biomarkers, such as in ultraviolet-visible spectroscopy (Alamprese *et al.*, 2013), FTIR or Fourier transform infrared spectroscopy (Rahmania *et al.*, 2015), chromatographic-based techniques (Chou *et al.*, 2007), and protein-based tests like enzyme-linked immunosorbent assay (ELISA) using a specific immunoassay (Asensio *et al.*, 2008; Rao *et al.*, 2016). In certain conditions during food processing, these biomarkers are subject to extensive breakdown or degradation (Ali *et al.*, 2018; Karabasanavar *et al.*, 2014).

DNA-based identification of meat species has evolved as the most commonly used method because it offers several major advantages, including fast and reliable detection, cost efficiency, and suitability for in situ detection systems, especially after the 2013 “Horse Meat Scandal” in parts of Europe. Undeclared or improperly declared horse meat was found in foods advertised as containing beef (O’Mahony, 2013). In the context of meat species detection, the application of DNA has resulted in accurate confirmation and identification of meat present in numerous types of foods, regardless of how the meat-based food has been processed because DNA is more resistant and thermostable than protein (Cravero *et al.*, 2019). DNA is a very stable and long-lived biological molecule that can be retrieved from any substrate because it exists in almost all cells of an organism (Mafra *et al.*, 2008).

Polymerase chain reaction (PCR) and its development, such as real-time PCR, multiplex PCR, TaqMan real-time PCR, are among easily performed DNA-based techniques commonly used for halal-meat authentication. Real-time PCR using primers that are specific for certain species has been used to detect non-halal meats, e.g., pork (Orbayinah *et al.*, 2019), wild boar meat (Widyasari *et al.*, 2015), rat meat (Aina *et al.*, 2019), dog meat in meatball products (Aina *et al.*, 2019), and cat meat in burgers (Ali *et al.*, 2015). In this study, pork and wild boar meat are identified using a newly designed primer targeting mitochondrial DNA.

MATERIALS AND METHODS

Materials

Experimental materials

Wild boar meat (WBM) was obtained from several hunters in Baamang District, Kotawaringin Timur Regency, the Province of Kalimantan Tengah, Indonesia. Meanwhile, pork was obtained from Godean District, Sleman Regency, D.I. Yogyakarta Province, Indonesia. As for other meats, they were collected from several regions in Yogyakarta, Indonesia: beef, goat meat, and chicken were purchased from Mlati District in Sleman, Yogyakarta, rabbit meat was obtained from Kaliurang District in Sleman, Yogyakarta, and dog meat was obtained from Bantul Regency in Yogyakarta, Indonesia.

Primer design

The forward (F) and reverse (R) primers of NK-ND1-Ssc1 were designed *in silico* using PrimerQuest software from Integrated DNA Technologies. The complete DNA sequence and the ND1 subunit of the mitochondrial genome (AP003428.1) of *Sus scrofa domestica* used for primer design were retrieved from NCBI GenBank. The primer specificity was checked *in silico* using NCBI Primer-BLAST and compared with beef, goat meat, chicken, rabbit meat, and dog meat. The designed primers (NK-ND1-Ssc1) were:

Forward: AAA GGA CCC AAC GTT GTA GG

Reverse: TAG TGC TAG GGA TAA GGC TA GG

These primers have a melting temperature of 57.84°C (F and R) and GC contents of 50% (F) and 50% (R) with an amplicon size of 147 base pairs (bp) in length.

Methods

The extraction and evaluation of DNA

The DNAs used for PCR assay were extracted manually using a mixture of phenol:chloroform:isoamyl alcohol (PCIA) according to Sambrook *et al.* (1989). Approximately 200 mg of the evaluated sample was placed in sterile 2 mL reaction tubes and added with 800 µL of buffer lysis (consisting of EDTA pH 8, Na-acetate pH 5.2, Tris HCl pH 8, NaCl, and 1% SDS), 30 µL of 20 mg/mL proteinase K, and 0.2 mL of buffer lysis. Then, this mixture was vortexed (Barnstead, USA) for 5 min, incubated in a water bath (IK HB 10, Medford, USA) at a temperature of 55°C for 60 min with occasional shaking (15 min each). The suspension was centrifuged (5 min, 13,000 gm) in a microcentrifuge (Sartorius 3-30K Sigma, Germany). The supernatant (400 µl) was pipetted off to a new tube and added with 200 µL of cold phenol (Merck, Darmstadt, Germany), creating a homogenous mixture. This solution was then shaken with a shaker in an incubator (Biobase, China) for 30 min, and the suspension was centrifuged at 13,000 xg for 10 min. The resultant supernatant was removed and subsequently added with chloroform (1:1 v/v) and subjected to centrifugation at 13,000 xg for 10 min. DNA was sedimented with 2.5 M Na-acetate pH 5.2 (1:0.1 v/v) and cold absolute ethanol (1:2 v/v). The DNA obtained was then washed with 0.25 mL of 70% ethanol, dried, and added with 50 µL of Tris-EDTA buffer (consisting of 10 mM Tris HCl pH 8 and 1 mM EDTA). The extraction was performed two times.

Amplification analysis of DNA using PCR

The extracted DNA was analyzed using qPCR. The reagent (20 µL) was composed of 10 µL of EvaGreen qPCR master mix, 1 µL of the forward primer and reverse primer, 1 µL of DNA template (50 ng/µL), and 7 µL of nuclease-free water. The conditions used were: pre-denaturation at 95°C for 30 sec (1 cycle), denaturation at 95°C for 5 sec, annealing at an optimized temperature of 58.1°C for 30 sec, extension or amplification at 72°C for 10 sec (cycled for 25 times), and elongation at 72°C for 5 min to stabilize the amplification product.

Evaluation of PCR products

The PCR products were visualized through electrophoresis (i-Mupid J Cosmo Bio Co., Tokyo, Japan) using 2% agarose in TBE 1x for 30 min at 100 V to examine whether the designed SSP had successfully amplified the DNA. In this test, 4 µL of each type of DNA with a concentration of 50 ng/µL was used. The result was recorded using a transilluminator (Bio-Rad, USA). The resultant band was compared with the DNA marker to determine the size of the amplicon. The DNA marker used was 100 bp DNA ladder (GeneAid, Taiwan) (Orbaniyah *et al.*, 2019).

Evaluation of reference samples of sausages

The reference samples used in this study were sausages made from chicken, pork, and wild boar meat (WBM). First, fresh chicken meat (CM), pork (PM) and WBM were washed and drained. Second, the meats (CM, CM-PM-WBM, PM-WBM) were prepared to comprise as much as 90% of the sausages and, then, mashed and mixed with wheat (10%), salt (0.01% wt/wt), and other herbs commonly used in making sausages. This mixture was then wrapped with sausage casing and then cooked in boiling water. The DNA of the reference sausage was extracted and then tested using qPCR. The qPCR methods used were conditioned according to the manufacturer's procedure given (Biotium Inc., Bio-Rad Laboratories, CA, USA). In a reaction tube, 10 µL of EvaGreen, 7 µL of nuclease-free water, 1 µL of 10.0 µM forward primer, 1 µL of 10.0 µM reverse primer, and 1 µL of extracted DNA (50 ng/µL) were mixed and analyzed using an instrument (real time-PCR, CFX96 Touch Real-Time PCR Detection System from Bio-Rad, the United States). The qPCR procedure was as follows: pre-denaturation at 95°C for 30 sec (1 cycle), denaturation at 95°C for 5 sec (25 cycles), annealing temperature at 58.1°C for 30 sec, and extension or amplification temperature at 72°C for 10 sec. The relative fluorescence signals were read at the end of each cycle. In the melting curve analysis, the temperature was set at 65°C–95°C with an increment of 0.5°C/5 sec. The evaluation included linearity, efficiency, and sensitivity (Bio-Rad, 2006). The data were processed and analyzed in CFX Maestro™ software, which is included in the qPCR instrument.

Validation of real-time PCR for quantitative analysis

The real-time PCR using the primers of NkND1-Ssc-1 was validated based on the guidelines introduced by the Codex Alimentarius Commission, that is, by assessing specificity, linearity, sensitivity (represented by limit of detection and limit of quantification), amplification efficiency, and precision (represented by the repeatability of the developed method). The validated method was then applied to the identification of DNA in commercial meatball samples.

Data Analysis

The real-time PCR results were analyzed using a standard curve created by comparing C_q values (Quantification circle) with the log-transformed concentration of DNA. Meanwhile, the real-time PCR efficiency (E) was calculated using the formula: $E = (10^{-1 / \text{slope}} - 1) \times 100$ (Bio-Rad, 2006).

RESULTS AND DISCUSSION

Purity and concentration of extracted DNAs

Extraction of DNA from raw meats or sausage samples is the first step for the successful identification of meat species intended for halal authentication. It can be carried out using the manual procedures of some DNA extraction methods or DNA extraction kits supplied by several manufacturers.

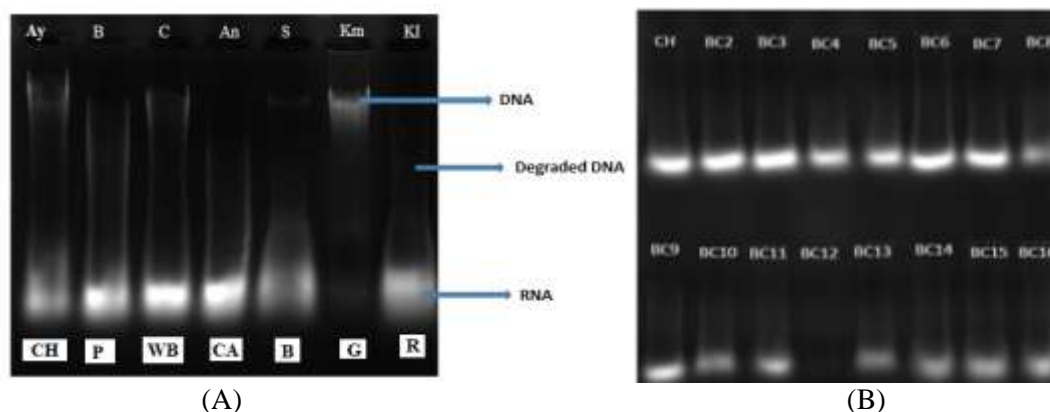


Figure 1. The agarose-gel electropherograms of DNAs isolated from raw meats. (A) shows DNAs of chicken (CH), pork (P), wild boar (WB) meat, dog meat (CA), beef (B), goat meat (G) and rabbit meat (R), while (B) shows DNAs isolated from the reference samples of sausages made from chicken (CH) and the combination of chicken, pork, and wild boar meat with varying compositions (BC 2-16)

Figure 1 shows the agarose-gel electropherogram of DNAs isolated from raw chicken, pork, wild boar meat, dog meat, beef, goat meat, and rabbit meat (A) and DNAs isolated from the reference samples of sausages made from chicken and the combination of chicken, pork, and wild boar meat with varying compositions. Table I compiles the purity levels and concentrations (yields) of DNAs extracted from several matrices. The extracted DNAs had ratios approaching 1.7-2.0.

Table I. The purity levels and concentrations of DNAs extracted from raw meats and reference samples of sausages containing chicken, pork, and wild boar meat

Samples	Ratio (260/ 280 nm)	Concentration (ng/ μ L)
<i>Raw meats</i>		
Chicken	1.80	3598.89
Pork	2.02	2350.00
Wild boar meat	1.97	2840.62
Dog meat	1.97	2566.28
Beef	1.45	2187.76
Goat meat	1.79	333.56
Rabbit meat	1.72	2537.02
<i>Sausages containing</i>		
CM without PM-WBM (0%)	1.95	1391.19
CM-PM-WBM (0.1%)	1.79	1580.29
CM-PM-WBM (0.3%)	1.30	3180.84
CM-PM-WBM (0.5%)	1.13	3437.21
CM-PM-WBM (0.7%)	1.15	3189.88
CM-PM-WBM (0.9%)	1.26	3131.77
CM-PM-WBM (1%)	1.76	234.84
CM-PM-WBM (2%)	1.18	3364.38
CM-PM-WBM (3%)	1.13	3333.17
CM-PM-WBM (5%)	1.31	3185.64
CM-PM-WBM (10%)	1.65	149.78
CM-PM-WBM (25%)	1.10	3106.08
CM-PM-WBM (50%)	1.11	3061.35
CM-PM-WBM (75%)	1.64	1569.25
PM (100%)	1.62	2259.71
WBM (100%)	1.64	3155.80

Validation results of real-time PCR

Figure 2 depicts the amplification curves and denaturation peaks of pork and wild boar meat using primer at various annealing temperatures (T_a). The highest relative fluorescence unit (RFU) during the amplification of DNAs of pork and wild boar meat was obtained at T_a of 58.1°C

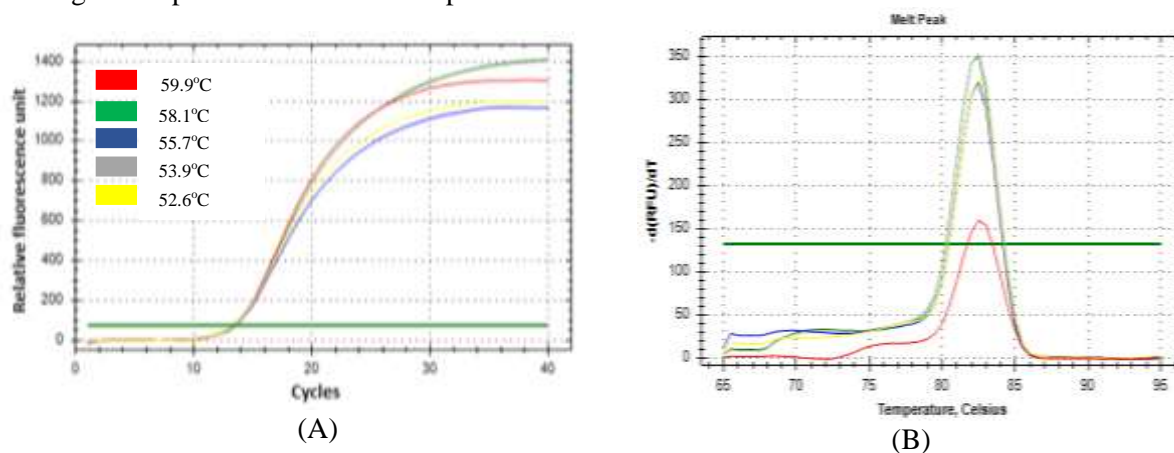


Figure 2. The amplification curves and denaturation peaks of DNA extracted from pork using the primers of NK-ND1-Ssc1 at various annealing temperatures: 59.9, 58.1, 55.7, 53.9 and 52.6°C

Figure 3 shows that the designed primer could amplify DNAs isolated from pork specifically at a melting temperature (T_m) of 82.50 and WBM at T_m of 83.00 but not DNAs of nontarget species. According to this graph, the annealing temperature of 58.1°C can be used in the validation test.

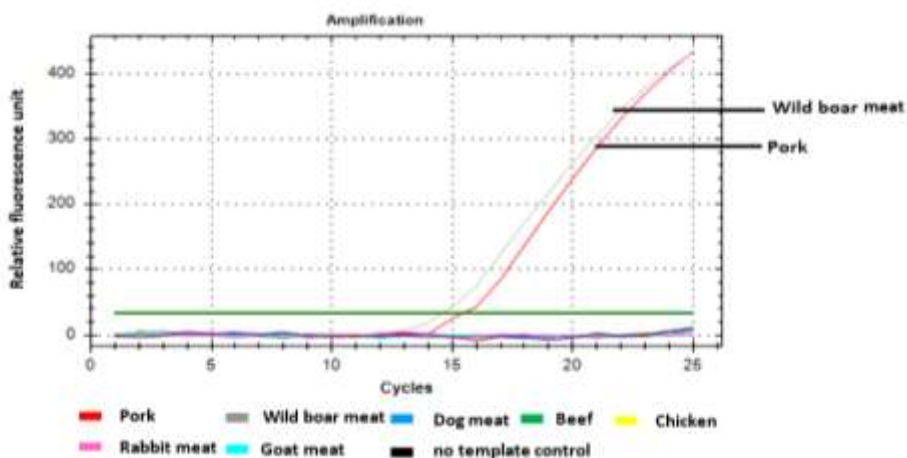


Figure 3. The amplification curves of DNAs isolated from pork, wild boar meat, dog meat, beef, chicken, goat meat, rabbit meat, and no template control (negative control) using the primers of NK-ND1-Ssc1 at an annealing temperature of 58.1°C

The results of the sensitivity and efficiency tests are shown in Figures 4 and 5. The DNAs extracted from pork and WBM could be distinguished and amplified when present at the lowest concentration of 50 ng; therefore, the limit of detection was 50 ng. The linear regression analysis between the log values of porcine DNA concentrations resulted in a coefficient of determination (R^2) of 0.992 with an efficiency value (E) of 93.1%. As for the analysis of DNA extracted from wild boar meat, the R^2 and E values were 94.8% and 0.995, respectively.

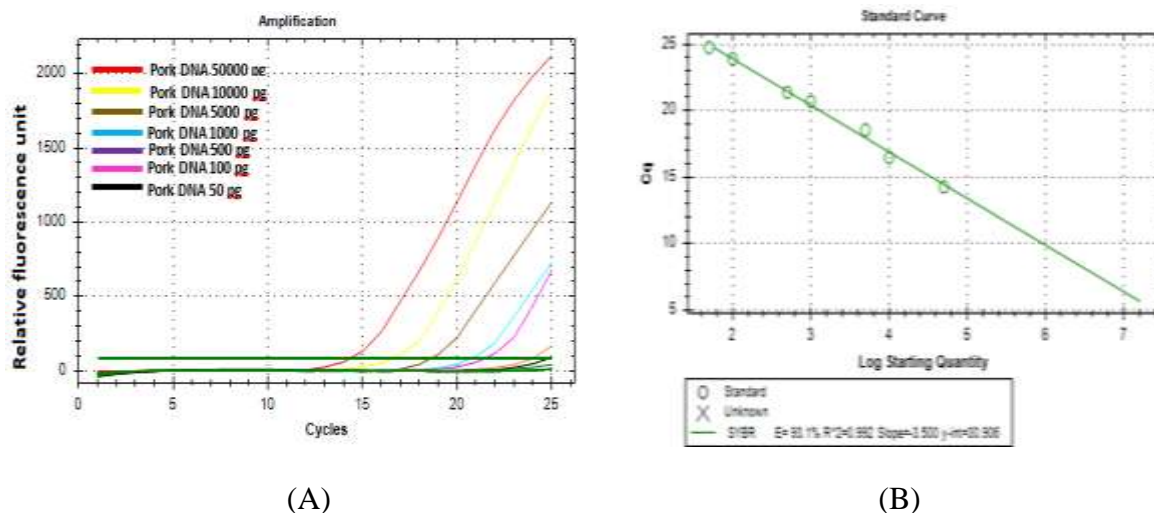


Figure 4. The amplification curves (A) and the corresponding linear regression (B) between porcine DNA concentration and cycle threshold (Cq) using the primers of NK-ND1-Ssc1 at an annealing temperature of 58.1°C

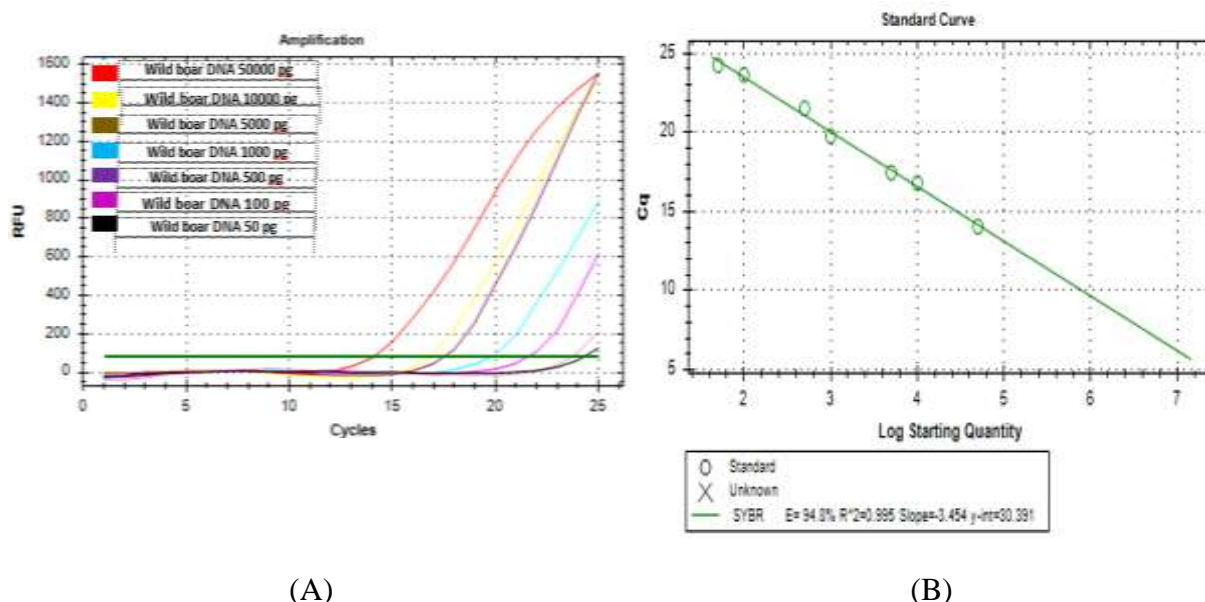


Figure 5. The amplification curves (A) and the corresponding linear regression (B) between the concentrations of wild boar meat DNA and cycle threshold (Cq) using the primers of NK-ND1-Ssc1 at an annealing temperature of 58.1°C

Application of validated methods to real sample analysis

Figure 6 presents the amplification profiles of DNAs extracted from commercially available sausages. All the evaluated samples did not contain pork and WBM in their formulation.

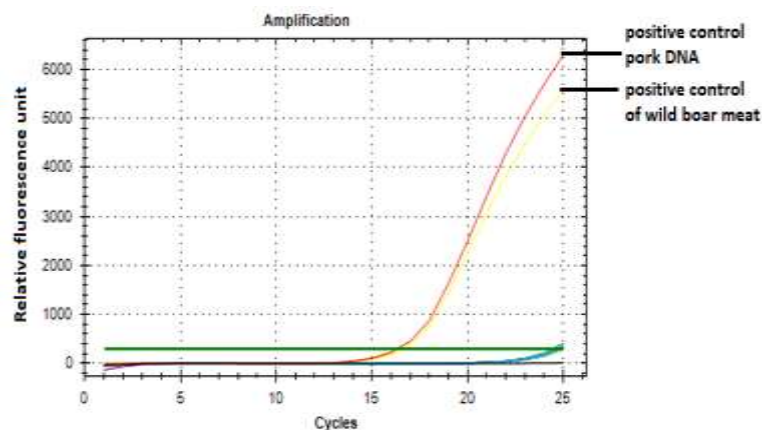


Figure 6. The amplification curves of DNAs extracted from samples of commercial sausages along with positive controls of DNAs extracted from chicken sausages containing pork and wild boar meat

In this study, the manual extraction system of phenol-chloroform-isoamyl alcohol (PCIA) is used for DNAs extraction. However, prior to use, it must be buffered to achieve pH 8.0 or above because DNA can be lost in an acidic condition. The use of SDS and proteinase K in this extraction system is intended to digest non-nucleic acid components and proteins enzymatically, while the mixture of PCIA is to partition cellular debris and lipid components into the organic phase (the isolated DNA remains in the aqueous phase). Isoamyl alcohol reduces foaming during the extraction process.

Electrophoresis using agarose gel is used to visualize or check the isolated DNAs. Fig. 1(A) shows that all DNAs can be isolated, as indicated by the appearance of DNA bands in agarose gel. However, not every fluorescence band is DNA; it may be RNA, protein, or degraded DNA. In Fig. 1(B), there are smears of DNAs isolated from sausages as a result of heat treatment during cooking, which degrades DNA. Therefore, the yield and purity of the isolated DNAs are necessary to identify. As seen in Table I, the extracted DNAs have high yields (ratios approaching 1.7-2.0), indicating that DNAs are enough to be used in real-time PCR analysis. Furthermore, DNAs with purity levels of far from 1.8-2.0 are not problematic because real-time PCR can amplify them, provided that the mixture of PCR reaction contains a DNA template even in a small quantity.

Before validating the real-time PCR, the conditions used to run it need to be optimized, preferably using annealing temperature. NK-ND1-Ssc1 is a specific primer designed *in silico* using NCBI Primer-BLAST (*Basic Local Alignment Search Tool*) targeting the chromosomal DNA of pork (*Sus scrofa domestica*), wild boar meat (*Sus scrofa*), beef (*Bos indicus*), chicken (*Gallus gallus*), dog meat (*Canis lupus familiaris*), goat meat (*Capra hircus*), and rabbit meat (*Leporidae*). The optimization of annealing temperature is essential in optimizing the attachment of the designed primer to the DNA template. The annealing temperature (T_a) is selected based on its capability to produce the highest signal (relative fluorescence unit, RFU) with the lowest cycles. The average annealing temperatures of the forward primer and reverse primer are estimated to be 57.73°C and 57.93°C, respectively. Therefore, some temperatures in the range of 53.9°C-59.9°C are preferable, namely 59.9, 58.1, 55.7, 53.9, and 52.6°C. Figure 2(A) displays the amplification curves and denaturation peaks of pork using primer at various annealing temperatures. Based on the highest RFU values (1200) and lowest cycles (14.92), the annealing temperature of real-time PCR for pork identification is 58.1°C, with the melting temperature (T_m) of 82.50.

The amplification of NK-ND1-Ssc1 primer for the detection of DNA of wild boar meat also uses the same annealing temperature as that of pork DNA. At T_a of 58.1°C, the highest RFU is 1400, with a quantification cycle (Cq) of 13.30 and T_m of 83.00, as shown in [Figure 2\(B\)](#). Furthermore, this T_a has been used in the specificity tests of DNAs extracted from wild boar meat (*Sus scrofa*), beef (*Bos indicus*), chicken (*Gallus gallus*), dog meat (*Canis lupus familiaris*), goat meat (*Capra hircus*), and rabbit meat (*Leporidae*).

NK-ND1-Ssc1 can be used to amplify DNAs isolated from pork, wild boar meat, dog meat, beef, chicken, goat meat, rabbit meat, and no template control (negative control) at an annealing temperature of 58.1°C, and the amplification curve produced is shown in [Figure 3](#). The forward and reverse primers can amplify DNAs isolated from pork and wild boar meat at T_m of 82.50 and 83.00, respectively, but cannot amplify DNAs from other meats. This means that the designed primers can specifically amplify the DNAs of pork and WBM simultaneously, which, in the case of halal authentication, provides an advantage because both types of meat are non-halal. As a consequence, using one single primer, both non-halal types of meat can be amplified, while DNAs from halal meats are not amplified.

The sensitivity of real-time PCR using the primers of NK-ND1-Ssc1 for detection of pork and WBM can be evaluated by determining the lowest concentrations of DNAs that can amplify the DNA templates, or termed limit of detection (LoD). The sensitivity is measured from the intercept of the linear regression between Ct or *Cycle threshold* (y-axis) and the log values of DNA concentrations (x-axis) from the serial dilutions of DNAs of raw pork and WBM at 50000 pg, 10000 pg, 5000 pg, 1000 pg, 500 pg, 100 pg, and 50 pg, as well as the serial dilutions of DNAs extracted from sausages containing 100% pork and 100% WBM. The amplification curves are shown in [Figures 5 and 6](#). DNAs of pork and WBM can be amplified at the lowest concentration of 50 ng, while, at a lower concentration (<50 pg), no amplified DNA is observable until the 30th cycles. Therefore, the LoD of DNAs of pork and wild boar meat using NK-ND1-Ssc1 is 50 pg ([Ali et al., 2013](#)).

The amplification efficiency of NK-ND1-Ssc1 can be assessed using the linear regressions presented in [Figures 4 and 5](#). The linear regression between Cq and the log values of porcine DNA concentrations shows a coefficient of determination (R^2) of 0.992 with an efficiency value (E) of 93.1%. Meanwhile, the R^2 and E values of the DNA of WBM are 94.8% and 0.995, respectively. All of which have met the qualitative and quantitative test criteria set by the [Codex Alimentarius Commission \(2010\)](#), i.e., $R^2 \geq 0.98$ and E of 90–110%. Based on the repeatability test results, the coefficient values (CV) of Cq are 0.22% and 0.50% for DNAs extracted from pork and WBM, respectively. The CV values fall within the acceptance criteria according to CAC ([Codex Alimentarius Commission/GL 74, 2010](#)). i.e., CV for real-time PCR $\leq 25\%$ ([Sow et al., 2017](#)). The real-time PCR method developed in this study has also been applied to the detection of pork and WBM in commercial chicken sausages. Twelve commercial samples collected from several areas in Yogyakarta, Indonesia, show no amplification. Also, [Figure 6](#) presents that only positive controls (DNAs extracted from chicken sausages containing pork and WBM) are amplified, meaning indicated that the evaluated samples of chicken sausages do not contain pork and WBM in their formulations.

CONCLUSION

Real-time PCR using NK-ND1-Ssc1, a designed primer, at an annealing temperature of 58.1°C has been successfully validated and used for identification of pork and wild boar meat in chicken sausages. The combination of real-time PCR and NK-ND1-Ssc1 can be used for the analysis of pork and WBM (non-halal meats), although it cannot differentiate pork from WBM. The developed method can be used as an alternative for halal authentication analysis.

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