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Low cost and comprehensive pork detection in processed food products with a different food matrix

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ABSTRACT The adulteration of processed beef-based meat products with pork is a sensitive issue in Indonesia. Therefore a simple, low cost, and accurate method is required for the detection of pork, so as to protect consumers from accidental consumption of adulterated meat. In this study, we developed a detection method for the low cost identification of pork in processed meat products. We used the cost-efficient *Taq* DNA polymerase, Dream*Taq* Green PCR master mix (2x), and duplex PCR method to recognize pork simultaneously with 18S rRNA detection. A positive control containing a pork gene inserted into pGEM®-T easy was prepared, along with a negative control. The results of the duplex PCR were used to assess its specificity, detection limit, and its ability to recognize pork in processed meat products with a different food matrix. 18S rRNA detection was for confirming DNA integrity of DNA extracted from the processed food, while the positive control confirmed that the reagents were working well and the negative control confirmed a non-contamination problem. Following this, the duplex PCR was optimized and the optimum concentration primer for duplex PCR detection was found to be 3 µM for pork and 0.2 µM for 18S rRNA. As little as 3.125 ng of the DNA template could be used to detect whether a sample contained pork. Of the nine commercial processed meat products tested, five were found to contain pork while four halal products showed no signs of pork. It can be concluded that duplex PCR is a simple, fast, sensitive, specific, and low cost method of detecting pork in processed meat products.

KEYWORDS 18S rRNA; duplex PCR; low cost; pork; processed meat

1. Introduction

Halal product is one of the priorities for Muslims in Indonesia. Islamic law prohibits Muslims from eating pork or its derivatives. Pork is one type of non halal meat that is often mixed in processed meat products of beef and chicken. For determining halal product, besides food that comes from unauthorized ingredients also needs to be considered on how it has been slaughtered, tools used for slaughtering, tools used to process and serve, additive substance, and packaging (Krishnan et al. 2017). The fact shows that many adulteration of processed meat products occur in Indonesia. Some processed meat products have not been certified halal or have given adulterated information about their composition. The duplex PCR that low cost and high accurate is of urgent importance as alternative method for implementation of food labeling regulations and product quality control.

Detection of pork in processed meat products has been developed with many methods using either protein or DNA molecules. The analysis with protein include immunoassay (Asensio et al. 2008; Kim et al. 2005), electrophoresis (Montowska and Pospiech 2007), and chromatographic (Przybylski et al. 2017). Identified pork based on protein are less effective for meat that has undergone high processing because protein can be denaturated by high temperature, high pressure, and other processing technologies (Soares et al. 2013). Recently analytical molecular biology, particularly DNA using the polymerase chain reaction (PCR) have received distinctive attention (Ghovvati et al. 2009), because DNA is relatively stable compared to protein or other molecules and cross reactions among targeted animals less occur than protein (Dalmasso et al. 2004; Jain et al. 2007; Ghovvati et al. 2009; Soares et al. 2013; Ali et al. 2015; Hou et al. 2015) so it can be applied in highly processed meat products (Al-Tamimi and Ashhab 2012). Multiplex PCR is one of PCR methods that can simultaneously amplify genes at one reaction. Those process amplifies some of DNA targets in samples using multiple primers (Kitpipit et al. 2014; Ali et al. 2015; Hossain et al. 2016; Kim and Kim 2017). Multiplex PCR increases efficiency and reliability for simulta-

neous analysis of various animal species (Dalmasso et al. 2004). In addition, multiplex PCR works on the target simultaneously which can reduce both costs and time (Matsunaga et al. 1999; Fajardo et al. 2010; Ali et al. 2015; Nguyen et al. 2016).

Many PCR methods use sequence variation of mitochondrial genes. The use of mitochondrial DNA (mtDNA) is intended because mtDNA has the higher copy number than nuclear DNA which thereby increases sensitivity in the PCR process. It has been reported that the multiplex PCR method for identification of various animal species uses mitochondrial DNA. Multiplex PCR assay uses mitochondrial ND5, ATPase, and cytochrome b (cytb) for the detection of five meat species forbidden in Islam to be eaten (Ali et al. 2015). Multiplex PCR targeting mitochondrial ND2, 16S rRNA, and cytb were an accurate method to distinguish the meat of ducks, pigs, bovines, and sheep in processed food products (He et al. 2015). Irine (2013) identified the meat of dogs, cats, and tigers using cytb in food products and cosmetics. Multiplex PCR has also been applied to foodstuff analysis for identification ruminants, poultry, fish, and pigs using mitochondrial 12S rRNA and 16S rRNA (Dalmasso et al. 2004; Ghovvati et al. 2009).

With the aim of providing a low cost, comprehensive, simple, and reliable method, we developed duplex PCR assay for pork detection in processed meat products with comprehensive pork detection. The novelty of this study, we developed duplex PCR methods with economic commercial polymerase, generated positive control (as confirmation that the reagents working well) for comprehensive pork detection, to avoid dubious and misinterpretation of analysis, internal control detection (using 18S rRNA as confirmation the integrity and no inhibitor were present in the DNA sample, avoiding from false negative result), and also negative control (as confirmation that there is no contamination occurred in the chemical reagents).

2. Materials and methods

2.1. Preparation of meat samples

Raw meat samples (pork *Sus scrofa*, beef *Bos taurus*, duck *Anas platyrhynchos*, fish *Atule mate*, chicken *Gallus gallus*, goat *Capra aegagrus*) for a specificity test were purchased from either local markets (pork, beef, goat) or supermarkets (duck, fish, chicken) in Bogor, West Java. Processed food samples (N = 9) were purchased from a local market in Bogor. All samples were cut into small pieces, kept in a sterile plastic bag, labeled, and stored at -20°C until they were used.

2.2. DNA extraction

DNA extraction of raw meat samples using the PureLink® Genomic DNA Mini Kit (Invitrogen, Waltham, USA) followed the manufacturer's instructions, as did the DNA extraction of processed food using the DNeasy mericon Food Kit (Qiagen, Hilden, Germany). DNA was purified by the SV Gel wizard kit and the PCR-clean up system from Promega (USA). Purity and concentration of extracted DNA were measured by using Genequant spectrophotometer with the ratio of 260/280 nm.

2.3. Oligonucleotide primer

Two specific primer pairs used for duplex PCR amplification are listed in Table 1. The target genome of pork were designed from region of mitochondrial DNA (cytochrome b). This primer were published by Desriani and Widyowati (2017). The target site for internal control consisted of 99 bp fragment of the 18S rRNA that the conserved region of mitochondrial genes in all vertebrates (Martín et al. 2009).

2.4. The PCR specificity

The specificity were tested using DNA extracted from pork, beef, goat, fish, duck, and chicken, respectively. PCR reaction was performed with a total volume of 10 µL. Amplification uses Dream*Taq* Green PCR master mix (2x) kit by Thermo Scientific. Amplification of the gene segment was performed by the simplex PCR method with the thermo cycler machine with the condition: pre-denaturation at 95°C for 5 min; 35 cycles were programmed with denaturation 95°C for 30 s, optimized annealing at 55°C, 57°C, 60°C, 61°C, 62°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min. The amplification products were visualized on 2% agarose gel.

2.5. Preparation of positive control

Cloning was performed using the pGEM®-T Vector System kit by Promega (USA). Pork specific gene extracted from pork genomic DNA further inserted into those plasmid. The DNA insert into plasmid was confirmed by PCR colony and DNA sequencing.

TABLE 1 Oligonucleotide used as species-specific primer.

Sequence 5'→3'	Amplicons (bp)	Reference
Pork-Fwd: AAGAATATCCACCACACGAA	300	Desriani et al. 2017
Pork-Rev: GGTTCAAGTACCCATACAT		
18S-Fw: AGGATCCATTGGAGGGCAAGT	99	Martin, et al. 2009
18S-Rev: CCAACTACGAGCTTTTTAACTGCA		

2.6. The optimization primer concentration of duplex PCR

The optimum concentration was determined because the optimal conditions of the polymerization reaction of each DNA fragment is different. First, we used concentration of pork primer 0.2 µM. Second, concentration of pork primer 0.8 µM and the last formulation used concentration of pork primer 3 µM. While concentration of 18S rRNA primer 0.2 µM. PCR assay was performed by Thermo Scientific Dream*Taq* Green PCR master mix (2x) kit with predenaturation at 95°C for 5 min, denaturation at 95°C for 15 s, annealing at 55°C for 30 s, extention at 72°C for 1 min, and final extention at 72°C for 5 min with 35 cycles using Supercycler SC-200 Kyratex. The PCR product was visualized by electrophoresis on 2% agarose gel.

2.7. The detection limit of duplex PCR test

To test the detection limit of duplex PCR serial dilution of pork DNA 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, and 0.78125 ng of DNA template were added in the respective reaction. The DNA band pattern should show two bands corresponding to the two target genes (pork and 18S rRNA).

2.8. Duplex PCR on processed meat products

With the optimized duplex PCR methods we tested one of processed meat product as our preliminary test. Furthermore we applied the optimized methods to nine processed meat products that have different food matrix. There were meatball, sausage, ham, corned beef, pasta, *abon*, and roulade. Five products contain pork and four products do not contain pork and assumed as halal products. DNA template from processed meat products was made with 20 ng DNA. Those PCR reactions were had complete and comprehensive confirmation by including positive control, 18S rRNA check, and negative control.

3. Results

3.1. DNA extracted

Concentration and purity DNA were measured by Genequant spectrophotometer. DNA has high purity if the ratio OD (optical density) at 260 nm and 280 nm has the value between 1.7 and 2.0 (Ali et al. 2015). If absorbance is more than 2.0, the tested solution still contains contaminants from the membrane protein or other compounds, so DNA content is not pure.

3.2. The PCR specificity

We confirmed the specificity of each primer to observe efficiency and evaluate pork primer set that only works on one of the target genes. The specificity of pork was performed using raw meat of six species that have been extracted. Primers were challenged in PCR with non-target species including pork, beef, duck, fish, chicken, and goat. We used the optimum annealing temperature which was 57°C that shown the target band was clear and clean (data were not shown). The primers were specific to target genes in this assay. Figure 1 shows primer worked on a specific fragment of 300 bp for pork. Negative control was not amplified in PCR reaction. Positive control was amplified according to expected DNA fragments (300 bp). The test was done twice with different days to avoid bias (Ali et al. 2015). While 18S rRNA primer could amplified in all of DNA template as expected (data were not shown).

3.3. The optimum primer concentration of duplex PCR

With concentration of pork primer 3 µM results in two amplified targets in each fragment length and the band of pork is thicker than the band of 18S rRNA. This formulation shows consistency testing that produces the same band pattern as the previous test. The result is the evidence of duplex reaction running well on DNA genome (Figure 2).

3.4. The detection limit of duplex PCR

After the test results with serial dilution obtained, the limit of PCR duplex detection was at the concentration of 3.125 ng DNA. In 3.125 ng DNA, both pork and 18S rRNA bands could still be amplified, although the band of 18S

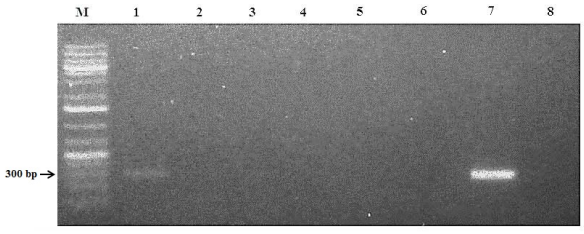

FIGURE 1 Specificity test with six target spesies and positive control. M: DNA ladder, 1: pork, 2: beef, 3: fish, 4: goat, 5: chicken, 6: duck, 7: positive control, 8: NTC (no template control).

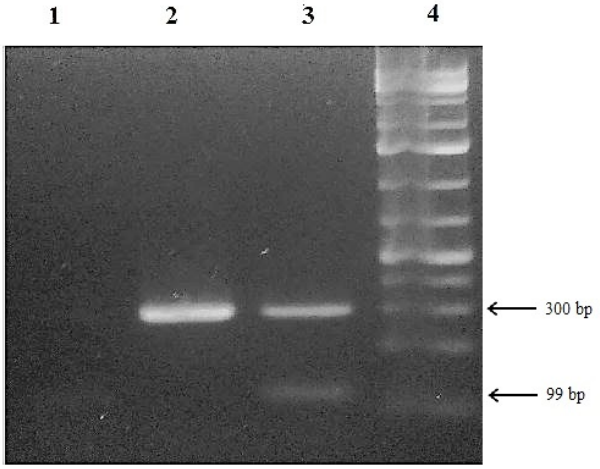

FIGURE 2 Duplex PCR in DNA genome. 1: NTC, 2: positive control, 3: duplex PCR, 4: marker.

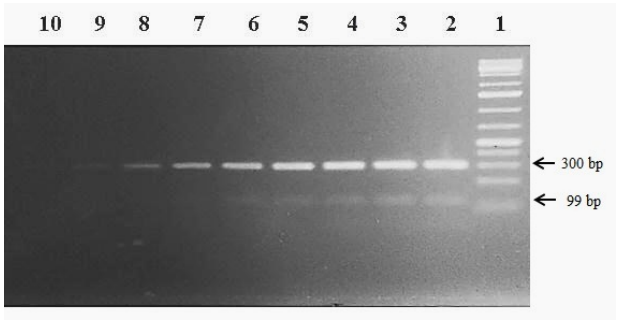

FIGURE 3 Sensitivity of duplex PCR in raw meats. Lane 1: marker, 2: 100 ng/µL, 3: 50 ng/µL, 4: 25 ng/µL, 5: 12.5 ng/µL, 6: 6.25 ng/µL, 7: 3.125 ng/µL, 8: 1.5625 ng/µL, 9: 0.78125 ng/µL, 10: NTC.

rRNA looked very thin. Figure 3 shows the result of amplification of two amplicons was detectable at the template as low as 3.125 ng DNA.

3.5. Duplex PCR on processed meat products

Concentration of duplex PCR DNA template was then optimized for pork detection on processed meat products. From three different concentration, we chosen 20 ng DNA template concentration that shown no different result as others (Figure 4). In this preliminary test, we can see the consistency of the duplex developed methods. This duplex method further were applied to nine different processed meat products that have different food matrix that was repeated twice. Result indicated that five samples are detected to contain pork, while the other four do not contain pork (Figure 5). This mean, the developed duplex worked as expected. The result was validated with positive control, 18S rRNA checked, and negative control confirmation. All of validation parameter worked well, so the dubious and misinterpretation result could be avoided.

4. Discussion

Detection of the fraud of halal processed meat products is priority in the country having the majority of the population as muslims to know the authenticity of products, to ensure the safety and halal of food ingredients, and to protect consumers from information falsification (Hidayat and Siradj 1999). Therefore very important to develop an accurate, high sensitivity, and also low cost method to simultaneously for detecting contamination of pork DNA because DNA contamination in processed food can occur in a very low level (Kim and Kim 2017). In this study, we developed a, simple, high sensitivity, low cost, and practical method to detection pork in processed meat products.

The specificity of a pair primers is the main points of successful amplication in PCR reactions (Matsunaga et al. 1999). The specificity primer was tested with five non target species (beef, chicken, lamb, duck, and fish) of the PCR system. The results showed a pair of specific-species primer of pork attached to a specific region, 300

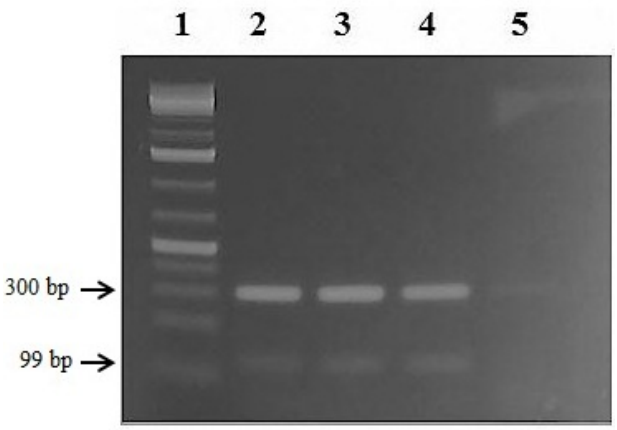

FIGURE 4 Duplex PCR in processed meat products with different concentration of DNA template. Lane 1: marker, 2: 66 ng/µL, 3: 50 ng/µL, 4: 20 ng/µL, 5: NTC.

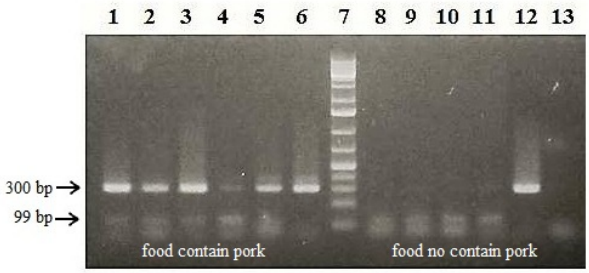

FIGURE 5 The result of duplex PCR in nine processed meat products. Lane 1: meatballs-1, 2: sausage, 3: ham, 4: pasta, 5: corned beef-1, 6: positive control, 7: marker, 8: corned beef-2, 9: *abon*, 10: roulade, 11: meatballs-2, 12: positive control, 13: NTC.

bp fragment length, in optimum annealing temperature at 55°C. On the other hand, the DNA template of non target species not showed the amplification band. Here we found a pair of specific-species primer that used has a high level of specificity because it was proven that only recognizes pork gene as a specific species target.

Duplex PCR is one type of multiplex PCR method that consists of two primers set to produce amplicon of size specific to different DNA sequences (Fajardo et al. 2010). Compared to single-species PCR systems (simplex), the multiplex PCR shows low operational cost, and increase speed, efficiency, and also reliability for simultaneous analysis of several animal species (Kim and Kim 2017). We optimized annealing temperature and primer concentration. The success of DNA amplification in the PCR process depends on the annealing temperature and the primer concentration (Markoulatos et al. 2002). The optimum PCR reaction makes sure the amplification of DNA and increases the quality of the PCR product (Joko et al. 2011). Optimum annealing temperature and primer concentration was obtained empirically. Results showed the best annealing temperature was 55°C with primer proportion of 3 µM for pork and 0.2 µM for 18S rRNA.

The detection limit of duplex PCR was evaluated using raw meat and processed meat products. The detection limit test in raw meat showed at concentration 3.125 ng DNA duplex PCR was run and both the target bands were still amplified although the band of 18S rRNA was very thin. Detection limit was important to observe the developed of duplex PCR performance in detecting the lowest concentration of DNA templates. In previously multiplex PCR assay reported that the detection limit of pork up to 0.2500 ng (Matsunaga et al. 1999), whereas Dalmasso et al. (2004) achieved 0.002 ng for the DNA template of pork. The difference of detection limit in multiplex PCR assay is reasonable phenomenon and it varies depend on target of species (Ali et al. 2015). With a different food matrix in processed meat products, we could clearly observe the duplex result at 20 ng DNA. In this concentration we got the clear and clean bands of DNA target. Duplex PCR was applied to nine processed meat products with a different food matrix and showed result variation. Five out of nine samples known contain pork with 300 bp amplicons for pork and 99 bp amplicons for 18S rRNA, in addition four samples are known no contain pork showed 99 bp amplicons of 18S rRNA. In Figure 5, the duplex PCR result shows consistency as the known ingredients.

The use of Dream*Taq* Green PCR master mix (2x) in this assay helps reduce costs. In our previous study, it has been also reported that the use of Dream*Taq* Green PCR master mix (2x) contributed to making of cheaper cost methods (Desriani 2014). As shown in Table 2, Dream*Taq* Green PCR master mix (2x) was cheapest of *Taq* polymerase. This *Taq* polymerase further has been reported to also be applied for some PCR routine check application (Charaya et al. 2016). However, as one of the commercial *Taq* polymerases, Dream*Taq* Green PCR master mix (2x) is susceptible to DNA contamination. Contaminant like bacterial DNA molecules has also been reported in several commercial *Taq* DNA polymerases, such as Dream*Taq* Green PCR master mix (2x) (Thermo Scientific); Go*Taq* Flexi DNA Polymerase (Promega); Mango*Taq* DNA Polymerase (BIOLINE); My*Taq* DNA Polymerase (BIOLINE); BioMix Red (BIOLINE); VELOCITY DNA Polymerase (BIOLINE); and Phusion High-Fidelity DNA Polymerase (Thermo Scientific) (Lupan et al. 2013). Some assays are reported provide DNase pre-treatment to reduce the occurrence of contamination (Heininger et al. 2003). Nevertheless, this would not be efficient and it would be costly.

TABLE 2 Comparison of *Taq* polymerases (University of Toronto 2018).

Polymerase	Price (Can $)	Total reaction
Dream*Taq* PCR Master Mix (2X) (Thermo Scientific)	95.96	200
2X PCR *Taq* MasterMix (Diamed)	111.10	200
2X PCR Master Mix with dye (Ambiogene)	127.86	200
HotStar*Taq* DNA Polymerase (Qiagen)	174.52	250
Platinum *Taq* DNA Polymerase (Invitrogen)	192.47	100

In this study, for avoiding DNA contamination problems in the PCR reaction we always keep all of the chemical reagent and the samples clean, dry, and cold. Furthermore to avoid all misinterpretation and dubious results as already mentioned above, we checked positive control, pork gene cloned (for confirming the reagent working well), internal control detection (18S rRNA check, for confirming the integrity of DNA sample), and also negative control (for confirming that no contamination exists in the chemical reagent). With this comprehensive check, it will increase accuracy for analyzing the result. Since we used also display positive control, internal control, and also negative control, all of dubious interpretation could be avoided. The duplex developed methods shown in this research activity indicated as simple, fast, sensitive, specific, and also low cost methods.

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Authors' contributions

DS, FAS, SY designed the study. DS, FAS wrote the manuscript. FAS, HW, MAW carried out the laboratory work. DS, FAS, SY, HW, MAW carried out data analysis. DS research funding. FAS, DS authors contributed to the final version of the manuscript. All authors discussed the results and contributed to the final manuscript.

Competing interests

We declare there are no competing of interests.

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