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Development and Validation of a Direct qPCR Method for the Detection of Pork Adulteration in Processed Meat Products

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Abstract

Background and Objective: The number of Muslims in Indonesia is increasing; hence, consumption of halal foods is increasing. This urges halal detection of foods as well. Direct qPCR is a method for DNA-based halal detection using crude DNA samples. One factor that affects the success of direct qPCR is the crude DNA. In this study, DNA extraction process was optimized by modifying the lysis step to achieve high-quality crude DNA, optimizing the qPCR conditions and validating the method.

Material and Methods: Materials used for DNA extraction from meats and meat products included lysis buffer containing EDTA, NaCl, SDS, NaOH and tween-20. Materials used in qPCR assay included crude DNA, ddH_20, primers and master mix. Genomes from meats and processed products were extracted using lysis buffer by optimizing lysis conditions (temperature and incubation time). The crude DNA was assayed for concentration and purity; then, results were compared to chloroform: isoamyl and the commercial methods. The DNA extract from lysis buffer was amplified using qPCR machine. Operational conditions such as DNA concentration, annealing temperature and primer concentration were optimized. Results were validated using specificity, repeatability, reproducibility, applicability, robustness and limit of detection assays.

Results and Conclusion: Temperature for optimum lysis in extraction lysis buffer method included 75 ˚C for 25 min. Concentrations of DNA from the lysis buffer, chloroform-isoamyl and commercial methods did not significantly vary (p-value=0.094). The optimum conditions for qPCR method were set at DNA concentration of 50 ng μl^{-1}, annealing temperature of 53 ˚C and primer concentration of 10 pmol. For the validation result, specificity assay showed that the method could be used to detect pork and wild boar in meats using ND4 primers. In addition, repeatability assay included 1.06% and reproducibility assay included 1.57%. Direct qPCR method can be used for various processed meat products and is resistant to inhibitors (alginate, calcium ions, EDTA and cellulose), except for polysaccharides. The LOD value achieved from the PCR sensitivity, linearity and efficiency assay were 0.001 ng μl^{-1}, 0.996 and 110%, respectively. Results verify that the direct qPCR method is easy, fast, applicable, accurate and precise for the pork detection.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Islam includes the second-largest population globally and is predicted to increase by up to 6.5% by 2050 [1]. Increases in the population increases consumption of halal foods as well, foods that do not contain pork and its derivatives, which is mandatory for Muslims [2,3]. Halal products can be detected using several techniques. One of the halal detection techniques is DNA-based, which includes advantages such stability at high temperatures and DNA detection in all organisms [4]. The qPCR technique is a DNA-based halal detection assay that is fast, specific and highly sensitive [5-7]. The qPCR detection results can be observed directly without the electrophoresis step [8]. Halal detection in meat using SYBR Green 1 qPCR has been carried out successfully

[9,10]. Detection of pork contents in processed meat products has been reported using real-time PCR [11-14].

A factor that affects qPCR detection method is the quality of DNA extract [8]. Extraction of the DNA in meats and meat products can be carried out using alkaline [15,16], cetyltrimethylammonium bromide (CTAB) [17] and the Phenol/chloroform/isoamyl [18,19] methods as well as commercial kits, which are developed based on those methods [20,21]. Technically, DNA extraction kits usually produce high concentrations and purity but are relatively expensive [15,22]. Conventional DNA extraction methods need a long processing time of approximately 24-48 h [23,24]. In Indonesia, the number of products that have received halal certificates is as little as 749,971 of all types of products. The number of business actors engaged in the food sector in the small micro-industries is nearly 4.21 million. This has yet to materialize, if the number of Halal Inspection Agencies (LPH) is compared with business actors or products that have not been halal inspected. Due to these conditions, a DNA extraction method that is fast, easy-to-use and inexpensive is needed.

Direct extraction methods using lysis buffer only can solve this problem. Lysis buffer is a compound that used to damage the cell wall; hence, DNA in the cell nucleus can be released. Use of lysis buffer produces crude DNA. Studies have been carried out to develop direct DNA extraction methods. Direct qPCR methods for agricultural products have been reported; however, no data are available using wild boar samples with species-specific primers [25]. Meat samples such as chicken, beef, mutton and pork were assessed using direct method with an average DNA concentration of 300 ng μl^{-1}; however, the resulting DNA purity was very small of 1.11-1.24 and amplification process was still carried out using conventional PCR methods [4]. Other researchers reported that three various lysis buffer formulations were used to extract porcine DNA with concentrations ranging 0.6-24.04 ng μl^{-1} and extraction process was carried out using sonication [26]. Of all the researchers who have carried out studies linked to direct PCR, the average concentration of DNA produced is still low and extraction process is carried out with additional equipment that not all the laboratories include. Furthermore, none of the samples in this study used pork and its processed products and have nor verified the pork detection process using qPCR. In fact, SNI ISO/TS 20224-3:2020 states that the detection of ingredients of animal origins in foods must be carried out using qPCR. Therefore, this study was carried out on the optimization and validation of pork detection methods using direct qPCR.

In this study, a lysis buffer method was developed by optimizing the lysis process at certain incubation time and temperature. The aim of development of this method was to increase success rate of the extraction process in various processed meat products. The resulting DNA extraction was amplified using ND4 primer designed in a previous study [27]. This primer could detect pork and wild boar with a detection limit (lowest DNA concentration 10^{-1} ng μl^{-1} using conventional PCR [27]. In addition, validation activities for qPCR were carried out immediately, including specificity, repeatability, reproducibility, applicability, robustness and LoD assessments. The ultimate goal of this study was to achieve a fast, inexpensive and accurate halal detection method using direct qPCR method. Optimization of DNA extraction conditions was investigated using lysis buffer, optimization of qPCR conditions as well as method validation.

2. Materials and Methods

2.1 Sample preparation

All samples were purchased from traditional markets in Malang, Indonesia. Pork (*Sus sucrofa domestica*), wild boar, beef, dog meat, rat meat, horse meat, chicken, rabbit meat, lamb and all meat products containing pork based on their labelling (ham, corned products, sausages, meatballs, candies, dendeng, crackers and abon) were used in this study. Each product was weighed as much as 5 mg and transferred into a 1.5-ml tube. Samples were stored at -20 ˚C for further use.

2.2 DNA extraction method

Three methods were used for DNA extraction, including kit and CI methods, compared with the lysis buffer method. The aim was to assess effectiveness of the lysis buffer method in extracting DNA. In addition, DNA extracts from all the methods were amplified using real-time PCR techniques and results were compared.

2.2.1 DNA extraction kit (from product)

The DNA extraction kit was produced from Exgene Clinic SV Mini (GeneAll, South Korea). Briefly, 50 mg of each sample were weighed, extracted and purified based on the manufacturer's instructions. Kit method was used as a comparative assay.

2.2.2 Chloroform-isoamyl (CI) method [28]

Chloroform-isoamyl method was carried out based on the method used in previous studies [14]. First, 50 mg of the sample were transferred into a 1.5-ml tube and mixed with 500 µl of STE buffer, 40 µl of 10% SDS and 20 µl of proteinase-K. This was vortexed for 20 s and incubated overnight at 55 ˚C and 800 rpm using thermomixer. Samples were centrifuged at 14000 g for 10 min at 29 ˚C and 400 µl of the supernatant were transferred into a fresh tube. A 1× volume of chloroform:isoamyl (24:1) was added to the mixture and agitated and then 40 µl of 5 M NaCl were added to the mixture. This was centrifuged at 14000 g for 10 min at 29 ˚C and 300 µl of the supernatant were transferred into a fresh tube. A 1× volume of chloroform:isoamyl (24:1) was added to the tube, agitated and re-centrifuged (14000 g, 29 ˚C, 10 min). Then, 200 µl of the supernatant were transferred into a fresh tube and mixed with 600 µl of cold absolute

ethanol and 400 µl of 5M NaCl. Tube was incubated at -20 ˚C for 2.5 h and centrifuged at 14000 g for 10 min at 4 ˚C. Pellets were mixed with 500 µl of 70% ethanol and centrifuged (14000 g, 4 ˚C, 5 min). Pellets were dried at 55 ˚C using thermomixer. Dried pellets were dissolved in 50 µl of buffer TE, pH 7.6.

2.2.3 Lysis buffer method (this study)

This method was developed by the current authors. Briefly, 5 mg of the sample were weighed and transferred into a 1.5-ml tube and mixed with 200 µl of lysis buffer (EDTA, SDS, NaOH, Tris-Cl and NaCl). Sample was incubated based on its treatment. Then, 45 µl of tween-20 were added to the mixture and centrifuged at 12,000 g for 10 min at 4 ˚C (patent no. S00202109696). Then, supernatant was transferred into a fresh tube.

2.3 Optimization of the lysis process (this study)

Optimization of the lysis process was carried out to optimize performance of the lysis buffer method. Optimization of the lysis process was carried out using a combination of temperature and incubation time. Combinations included A (95 ˚C, 5 min), B (85 ˚C, 15 min), C (75 ˚C, 25 min) and D (65 ˚C, 35 min).

2.4 DNA concentration and purity assay

The DNA extracts from the samples were calculated using NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA) to verify concentration and purity of the DNA extractx.

2.5 qPCR assay (from the products)

The qPCR amplification was carried out using 10 µl of reaction mixture consisting of 1 µl of forward primer (10 pmol μl^{-1}), 1 µl of reverse primer (10 pmol μl^{-1}), 1 µl of nuclease free water (Promega, USA), 5 µl of innuMIX qPCR DSGreen Standard (Analytik Jena, Germany) and 2 µl of the DNA extract. Primer was species-specific ND4 (patent no. S00202102169) (Table 1). Assessments were carried out using PCR program as follows: initial denaturation at 95 ˚C (2 min) and denaturation at 95 ˚C (30 s) and annealing/extension at 53 ˚C (1 min) for 40 cycles using qPCR (qTOWER3 G; Analytik Jena, Germany). Each assay was repeated three times.

2.5.1 Optimization of the qPCR process (this study)

Optimization of qPCR conditions included annealing temperature, DNA concentration and primer concentration. The annealing temperature included 51-55 ˚C. The primary concentrations consisted of 5, 7.5 and 10 pmol. The DNA concentrations included 1, 25, 50 and 100 ng μl^{-1}. The lowest Ct value included the best condition for the qPCR process.

2.6 Validation method (modified from [29])

2.6.1 Specificity assay

The assay was used to investigate if the primer could specifically recognize the target gene. Assessments were carried out using two DNA targets (pork and wild boar) and seven DNA non-targets (beef, dog, rat, horse, chicken, rabbit and lamb). A primer was considered specific if it could amplify the DNA target only.

2.6.2 Repeatability and reproducibility assays

Repeatability was carried out by an analyst to achieve similar results on each repetition. Assessments were carried out using samples of pork and wild boar extracts with seven replications as positive control and beef as negative control. Three analysts simultaneously carried out the reproducibility assay using samples of pork and wild boar. Each assessment was repeated five times. Results were analysed using t-assay to achieve the *p*-value. This method was insignificant if the *p*-value was greater than 0.05. The method was reported appropriate if the RSDR value was ≤ 25%.

2.6.3 Applicability assay

Ham, corned products, sausages, meatballs, candies, dendeng, crackers and abon were used as samples. All products included pork and sample extraction was carried out using lysis buffer method. The DNA extract was amplified using qPCR with three replications to assess its applicability.

2.6.4 Robustness assay

Assessments were carried out using several inhibitors, including polysaccharides, cellulose, calcium ions, EDTA and alginate. The inhibitory concentration was 1000 ng μl^{-1} per 50 ng μl^{-1} pork DNA. Amplification was carried out using qPCR with three replications.

2.6.5 Limit of detection assay

This assessment included sensitivity, efficiency and linearity assays. The aim of sensitivity assay was to investigate the smallest DNA concentration that could still be amplified. This assay was carried out by a concentration of pork DNA in a series of dilutions up to 10^{-10} ng μl^{-1}. An efficiency assay estimated the resulting amplicons, compared to the theoretical number of amplicons. A good efficiency value ranged 90-110% and a slope results from the standard curve of -3.1 to -3.5.

The efficiency formula was $E = 10^{(-1/slope)} - 1$. The aim of linearity assay was to investigate if two variables included a linear relationship. The best linearity assay results were greater than 0.98 [29].

2.7 Statistical analysis

The Ct values were generated by qPCR Real-Time Detection System Software v.4.1 (Analytic Jena, Germany). Then, concentration, purity of DNA and Ct values were analyzed using Minitab Software v.19 and Microsoft Excel v.2013.

3. Results and Discussion

3.1 Optimization of the lysis process

Differences in temperature and incubation time in the lysis process affected concentration and purity of the DNA extracts. Treatment A in the lysis process produced the highest DNA concentration of 386.11 ±26.61 ng μl^{-1}. High temperatures can damage membranes and cell walls; thus, further cell contents emerge [30]. The higher the DNA

concentration, the higher the contaminants extracted. Hence, the purity value decreases and the Ct value increases. The boiling treatment for 15 min on the extraction of bacterial DNA from milk produced as similar concentration and purity as conventional and kit treatments [31]. Statistical analysis showed that Treatment A significantly affected DNA concentration, compared to other treatments. This result indicated that three treatments of the cell lysis process did not include significant effects on DNA concentration. It was seen for the purity of DNA extract as well (Figure 1a). Purity of the DNA produced in all types of treatments was in the range of 1.50-1.56 or less than 1.80. It verified that the DNA was contaminated with proteins because the DNA extraction did not include a purification process. Protein contamination could decrease the A260/A280 ratio; hence, the resulting purity value was less than 1.70 [32]. Treatment C, including incubation temperature of 75 °C and an incubation time of 25 min, showed the lowest Ct value (Figure 1b.). It showed that the lower the Ct value, the more DNA was amplified. Therefore, the lowest Ct was used as reference for the best optimization treatment of DNA extraction [33]. Generally, 100% (ww^{-1}) pork mixed with beef included a Ct value of 16.4, while 1% (ww^{-1}) pork mixed with beef included a Ct value of 23.9 [20]. The DNA extract contaminants affected the Ct value and could interfere with the real-time PCR process. Contaminants could be detected in the sample matrix. The higher the temperature, the more cells are damaged. Thus, quantity of the contaminants is higher.

3.2 Comparison of various DNA extraction methods

The lysis buffer method included the lowest purity value (1.53 ±0.02), compared to the CI (2.01 ±0.04) and commercial kit (2.06 ±0.02) methods (Table 2). The lysis buffer method did not include purification steps; hence, purity of the DNA sample was relatively low. However, concentration achieved from the lysis buffer method was not significantly different from that from the commercial kit and CI methods (*p*-value = 0.094). The resulting Ct value was significantly affected by the DNA extraction methods (*p*-value = 0.000). However, the resulting Ct value was low and close to the kit and CI methods. The lysis buffer method can be used as an alternative DNA extraction method, which is faster, easier to use and inexpensive (Table 3).

Table 1: ND4 primer specifications

Primer	Sequence (5'-3')	Size (bp)	Number of nitrogenous base	Annealing temperature	Tm (^{0}C)	% GC
Forward	CCCATCCATCAATCTAATCGG	120	20	54 ○c	62	47.6
Reverse	ATGTAGAGAGAGTAGAGGGC		21		60	50

Source: [14]

Table 2. Concentration, purity and Ct value of DNA from various extraction methods DNA

Methods	Concentration DNA (ng μl^{-1})	Purity	Ct value
- Kit	304.86 ±18.31^a	2.06 ±0.02^a	11.55 ±0.06^a
- CI	203.17 ±86.17^a	2.01 ±0.01^a	12.43 ±0.33^b
- Lysis buffer	294.34 ±8.12^a	1.53 ±0.02^b	15.74 ±0.23^c

Note: Ct value with different letters in the same column are significantly different from each other

Table 3. Comparison of various DNA extraction methods

Description	Kit Method	Chloroform: isoamyl method	Lysis buffer method
Sample (mg)	50	50	5
Materials	- Buffer CL	- Buffer STE	- EDTA
	- Proteinase-k	- SDS 10%	- NaCl
	- Buffer BL	- Proteinase-K	- Tris-Cl
	- Etanol absolute	- Chlorofom	- NaOH
	- Buffer BW	- Isoamyl	- Tween-20
	- Buffer TW	- NaCl	- SDS 10%
	- Buffer AE	- Etanol absolute	
		- Etanol 70%	
	- Vortex	- Buffer TE	
Tools	- Thermomix	- Vortex	- Thermomix
	- Centrifuge	- Thermomix	- Centrifuge
	- SV column	- Centrifuge	
	- Mikropastle	- Mikropastle	
Time	18.5 hours	22 hours	50 minutes
Price	Expensive	Expensive	Cheap
Easy to use	Difficult	Difficult	Easy

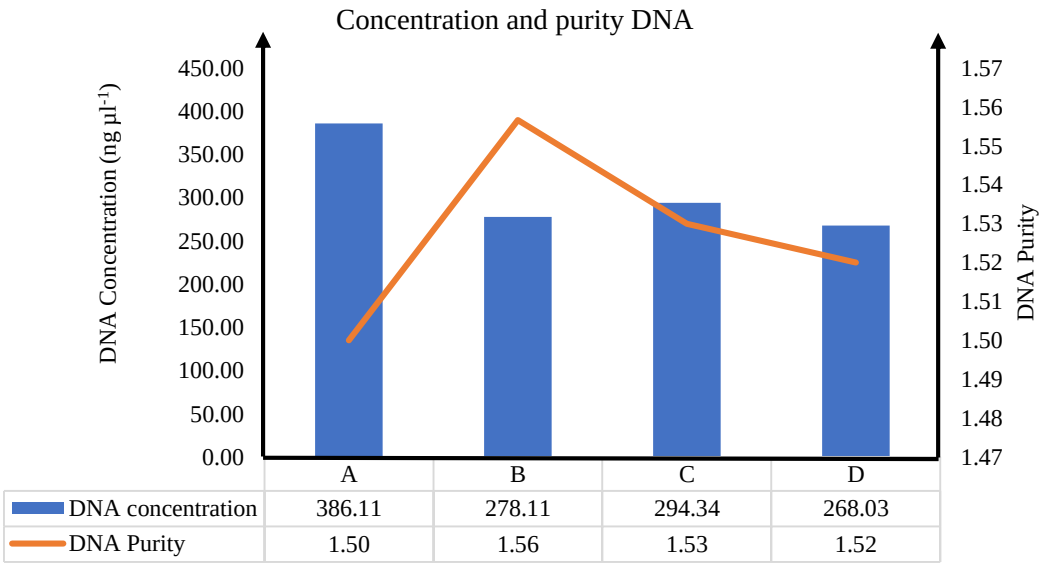

Figure 1a. Concentration and purity of DNA at various lysis conditions

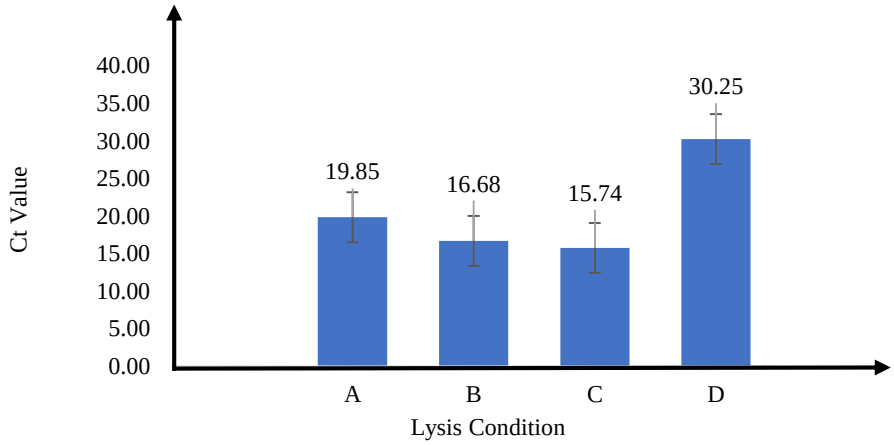

Figure 1b. Ct value at various lysis conditions

3.3 Optimization of the qPCR conditions

The lowest Ct value from the optimization process was 53˚C (Table 4). The higher the temperature, the higher the resulting Ct value. High temperature could break the hydrogen bonds between the base pairs in DNA; therefore, primers could not anneal appropriately [34].

Table 4. Optimization of annealing temperature

Temperature (°C)	Ct value
51	30.25
52	19.08
53	16.25
54	17.74
55	19.19

Note: Ct: *cycle threshold*

Low annealing temperatures caused the primer to anneal to a less specific area and amplified a small quantity of the target DNA, resulting in a high Ct value. The lowest Ct value was achieved using DNA at a concentration of 50 ng ul^{-1} (Figure 2). Dilution process could decrease the number of inhibitors involved in DNA and the quantity of intact DNA. As a result, quantity of DNA that could be amplified was smaller than the DNA with higher concentrations. Therefore, the resulting Ct value was high. In this study, the qPCR machine could not amplify DNA extract with a concentration of ≥ 100 ng μl^{-1}. The higher the DNA concentration, the greater the failure of the primer attachment. This was because inhibitors at high concentrations of DNA increased.

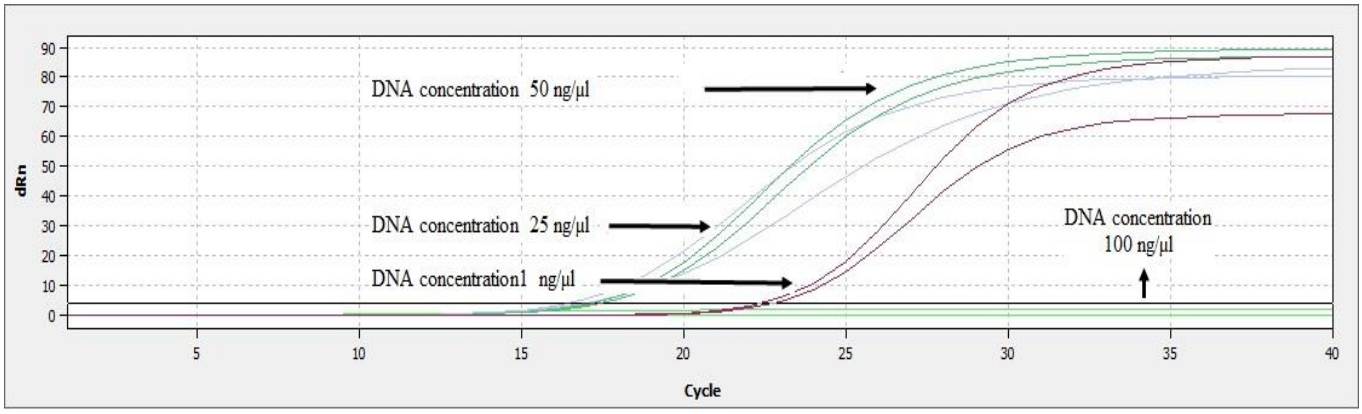

Figure 2. Amplification curve from the results of DNA concentration optimization assay

The best concentration of DNA used in the qPCR process was 50 ng μl^{-1}. Primer concentration could affect qPCR sensitivity. The ND4 primers produced the lowest Ct values at a concentration of 10 pmol (Table 5)

Table 5. Effect of various primary concentrations on Ct values

Primer concentration (pmol)	Average Ct value ±std
10	14.33 ±0.29
7.5	16.44 ±0.12
5	16.96 ±0.38

Note: Ct (*cycle threshold*)

The smaller the primer concentration, the higher the Ct value at a similar concentration of DNA. The higher the primer concentration, the more primers could attach to the DNA target. As a result, further DNA target could be amplified and hence the fluorescence could cross the threshold with faster results. Therefore, the primer concentration of 10 pmol was used as the best concentration for the amplification process.

3.4 Validation of direct qPCR

3.4.1 Primer specificity

One of the success factors of PCR was that the primer could attach specifically to the DNA target. The ND4 primer could amplify DNA targets, containing pork and wild boar samples (Figure 3). The amplification result was expressed in the Ct value, which could be read in the two samples. Specificity assessment on ND4 primers via conventional PCR method showed the presence of thick bands for pork and wild boar and produced a 120-bp amplification product of the DNA. Based on the results of *in-silico* analysis, the ND4 primer included a sequence length of 144 bp (located at 12338 to 12453 bp). Length of the sequence was verified as the sequence of ND4 in pigs (*Sus scrofa domesticus*). The ND4 gene in pigs was located at 11236 to 12607 bp [27]. In this study, the ND4 primer detected two DNA target types of pork and wild boar. Wild boars included similar DNA fragments of the pork DNA with 10% of their genomes [35]. Pork and wild boar included differences at the subspecies level and their genomes were homologous [36].

3.4.2 Method repeatability and reproducibility

Repeatability assay determines consistency of the tools and methods used to produce comparable data. This assay assesses precision of ~~a~~ data. This assay was carried out on two groups of samples, including positive samples (pork and wild boar DNA) and negative samples (beef DNA extract). All DNA samples (pork and wild boar) could be amplified (Table 6). The Ct value of all sample were relatively similar, with average values of 15.81 ±0.17 (pork) and 14.56 ±0.51 (wild boar). Results of statistical assays showed that each replication did not significantly affect the Ct value. Whereas in the negative control, all sample replications could not be amplified. This assay resulted in RSD_r values of 1.06% (pork) and 3.51% (wild boar). It verified that this assay included a high precision value since the RSD_r value of ≤ 25% was considered ~~as~~ high precision [29]. Several analysts carried out reproducibility assessment to verify that the resulting data were identical. The aim of the assay was to show accuracy of the assay. Assays were carried out by three analysts at the same time. All positive samples could be amplified with ND4 primers by all analysts (Table 7).

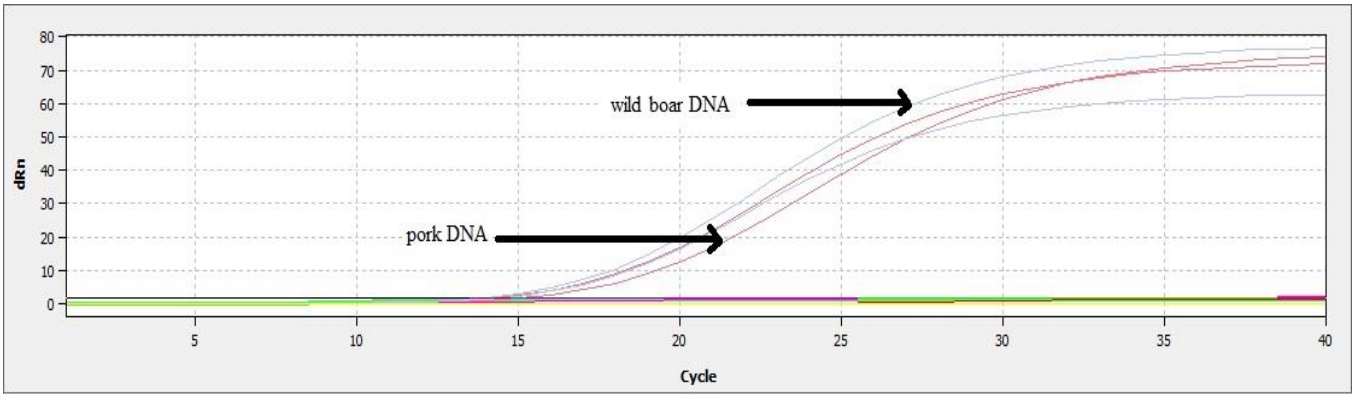

Figure 3. ND4 primer specificity curve

Table 6. Repeatability assay in the positive sample group using qPCR

Sample	Ct value	Tm value	Sample	Ct Value	Tm Value
- Pork	15.78[a]	74.10	- Wild boar	14.63[a]	74.30
- Pork	16.09[a]	74.00	- Wild boar	14.08[a]	74.30
- Pork	15.79[a]	74.00	- Wild boar	14.13[a]	74.20
- Pork	15.65[a]	73.80	- Wild boar	14.45[a]	74.20
- Pork	15.54[a]	73.60	- Wild boar	14.64[a]	74.00
- Pork	15.88[a]	73.60	- Wild boar	14.48[a]	74.00
- Pork	15.93[a]	73.80	- Wild boar	15.47[a]	73.90
Average	15.81	73.84	Average	14.56	74.13
Std	0.17	0.18	Std	0.51	0.15
RSD_r (%)	1.06	0.25	RSD_r (%)	3.51	0.20

Note: Ct value with different letters in the same column are significantly different from each other

Table 7. Reproducibility assay in pork and wild boar sample by three analyses

Sample	Analyst	Replicate	Average ± std	min –max Value	P-value
Pork	1	5	16.64 ± 0.09	16.52 – 16.75	0.064
	2	5	16.19 ± 0.27	15.69 – 16.93	
	3	5	16.64 ± 0.46	16.21 – 16.92	
Wild boar	1	5	14.32 ± 0.22	14.08 – 14.61	0.347
	2	5	14.62 ± 0.39	14.26 – 15.26	
	3	5	14.65 ± 0.79	14.12 – 15.11	

Note: each analysis does not give a significant difference to the results (p-value > 0.05)

Based on the statistical assays, *p*-value of the pork sample was 0.064, while the *p*-value of the wild boar sample was 0.347. The resulting *p*-value of greater than 0.05 on each assay by all analysts included no significant effects on the Ct value. This could be interpreted that the assays carried out by all analysts were able to produce relatively similar data; therefore, this method was accurate.

3.4.3 Method applicability

The lysis buffer method developed in this study successfully extracted all the processed meat products (Table 8). These results were similar to those of other studies that successfully extracted DNA from pork sausages, pork burgers, corned beef and pork meatballs using lysis buffer method (0.2 M Tris-HCl, 0.01 M EDTA, 0.5 M NaCl and 1% SDS) [25]. All total DNA concentrations were greater than 100 ng μl^{-1}. The highest DNA concentration was achieved from sausages with 685.21 ng μl^{-1}. On the sausage packaging label, the quantity of meat was 89%; therefore, it was suggested that a high concentration of pig DNA was produced. The higher the quantity of meat, the higher the concentration of DNA. However, purity of the resulting DNA was less than 1.80. This was because the DNA extraction process was carried out in one step (cell lysis with no purification steps). All DNA extracts could be amplified by showing Ct values. The lowest and the highest Ct values were achieved for ham (18.87 ±0.57) and abon, respectively. Low Ct values indicated presence of intact (not damaged) target DNA in the extracts with large quantities.

3.4.4 Method robustness

Efficiency of the qPCR method could decrease if inhibitor were available. Inhibitors could originate from a food product matrix such as alginate (emulsifier agent), EDTA (chelating agent), potassium ion (hardening agent), cellulose (stabilizer agent) and polysaccharides (stabilizer agenta). Polysaccharides (xanthan gum) in DNA extract significantly affected the Ct value from other samples (Table 9).

Table 8. Concentration, purity, and Ct value of DNA extract from various food products

Products	Concentration DNA (ng µl^{-1})	Purity	Ct value ±std
- Corned	413.90	1.14	32.46 ±0.14
- Meatball	263.08	1.61	32.79 ±1.40
- Sausage	685.21	1.14	19.87 ±0.30
- Crackers	264.88	1.70	32.88 ±0.68
- *Dendeng*	237.03	1.76	26.29 ±7.72
- Ham	482.96	1.73	18.87 ±0.57
- Candy	573.12	1.34	27.47 ±0.10
- *Abon*	549.86	1.43	33.70 ±0.52

Table 9. Effect of inhibitor on Ct value

Inhibitor	Sample	Type of inhibition	Ct value ±std
- Positive control	Pork DNA	-Taq DNA polymerase	15.74 ±0.18^{a}
- Alginate	Alginate	DNA	16.54 ±0.69^{a}
- Cellulose	CMC	Taq DNA polymerase	16.02 ±0.47^{a}
- EDTA	Na_2EDTA	Taq DNA polymerase	15.66 ±0.52^{a}
- Calcium	$CaCl_2$	Taq DNA polymerase	15.80 ±0.16^{a}
- Polysaccharide	Xanthan gum		17.78 ±0.56^{b}

Note: inhibitor with different letters in the same column are significantly different from each other

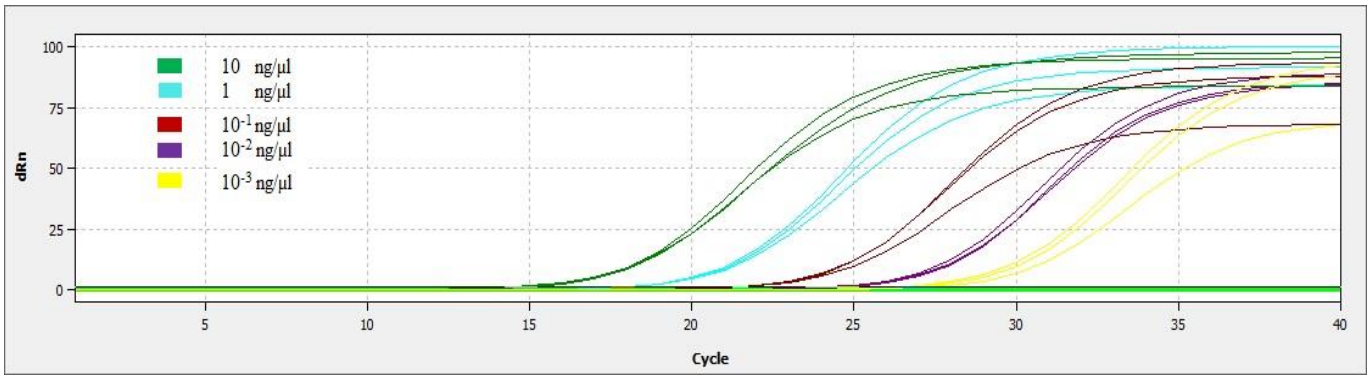

Figure 4. Amplification curve of pork DNA sensitivity assay

It verified that polysaccharides acted as inhibitors in the amplification process, resulting in a higher Ct value than that of the control and other samples. Polysaccharides could inhibit performance of Taq DNA polymerase [25].

3.4.5 Limit of Detection

The smallest DNA concentration that could be amplified was 10^{-3} ng µl^{-1} (Figure 4). Sensitivity value of the direct qPCR method was higher than that of the conventional PCR method. Concentration of DNA that could be amplified via the conventional PCR method and ND4 primer was 10^{-1} ng µl^{-1} [27]. It showed that the direct qPCR method was more sensitive than conventional method. The limit of detection (LOD) value assessment was based on linearity and efficiency calculations using standard curve. Acceptability of the linearity value (R^2) for quantitative assays was ≥ 0.98, with a range of slope values between -3.6 and -3.1 and PCR efficiency values between 90 and 110% (37). In this study, the resulting linearity value was 0.99, the slope were -3.1 and the PCR efficiency was 110%.

4. Conclusion

Direct qPCR is a halal detection method based on crude DNA, which is amplified in a qPCR machine. This method includes the advantage of being faster and less expensive than other methods [4]. However, this method includes the disadvantage that the resulting DNA purity is low due to the absence of a purification process [26]. In direct qPCR, lysis buffer is used for the process of lysing cells. The lysis buffer method includes EDTA (chelating agent), Tris-Cl (as a buffer), NaOH (damaging cell membranes), NaCl (precipitating proteins) and SDS (dissolving cell membranes) that can maximize DNA extraction [38].

The DNA concentration resulting from the lysis buffer method included no significant effects on other extraction methods of chloroform-isoamyl and commercial kits. However, the highest DNA concentration resulted from the kit method with 304.86 ±18.31 ng µl^{-1} (Table 2). The lysis process of the kit method was carried out overnight; hence DNA in the sample could be extracted optimally. In addition,

the sample weight was 50 mg, which was much more than that of the lysis buffer (5 mg) and the chloroform-isoamyl (20 mg) method. The larger the sample, the greater the surface area. Therefore, the extracted DNA was greater. As a result, DNA concentration was high. In addition, long lysis process allowed DNA extraction optimally. The cell lysis process carried out for 12 h was better than the lysis process for 2 h and 10 min as shown by the formation of thicker DNA bands [39]. The lysis buffer method included a high concentration of DNA of 294.34 ±8.12 ng μl^{-1}. This was because the temperature used in the lysis process was higher than that of the chloroform-isoamyl and commercial kit methods. Use of higher temperatures could affect permeability of the cell membranes. When permeability of the cell membrane was disturbed, molecules from outside the cell could easily enter the cell. As a result, the lysis process in cells could easily occur and DNA could be extracted appropriate [30]. In addition, high temperatures denatures proteins bound to the cell membrane; thus, cell membrane cannot function and was lysed [40]. The DNA extraction carried out using the lysis buffer method on various raw meats could produce DNA concentrations of 300-400 ng μl^{-1} [4].

The lysis buffer method included the smallest DNA purity of 1.53, compared to other methods. This was seen because this method did not carry out purification stages as in the chloroform-isoamyl and commercial kit methods. DNA purity achieved from the lysis buffer method was 1.20-1.28 [4]. In addition, the highest Ct value of 15.74 was achieved from the DNA extract using the lysis buffer method. This was occurred because purity of the resulting lysis buffer method was low. Therefore, contaminants were still detected in the resulting DNA extract. These contaminants could play roles in inhibiting the qPCR process; hence, the resulting Ct value was high (41). The Ct value produced from the pork samples using DNA lysis buffer extract was 33.82 [26].

The crude DNA resulted from the lysis process was successfully amplified using the qPCR method. Success of the amplification shown by the Ct value indicated effectiveness of the lysis process. The Ct value produced by each food product was different because each food product included different compositions and processing steps. The more complex the composition of the ingredients for processed meat products, the more inhibitors involved in the DNA extraction process. The greater the number of inhibitors, the more inhibition of the qPCR, making the Ct value higher. For example, polysaccharides in meatballs could bind to DNA polymerase and hence the elongation process could not be carried out appropriately [41]. The processing process could also affect DNA concentration and value of Ct. The higher the processing temperature; the more degrade of DNA. As a result, the resulting DNA was not intact and thus it could not be maximally amplified [42].

5. Acknowledgements

In this study, the optimum conditions for the lysis process in DNA extraction with lysis buffer were 75 °C and 25 min. Crude DNA from the lysis buffer process included concentrations that were not significantly different from DNA extracted with chloroform-isoamyl and commercial kit methods. Moreover, purity of the DNA was lower than that of the two methods because no purification process was used. Crude DNA was amplified using qPCR machine with the best annealing temperature of 53 °C, primer concentration of 10 pmol and DNA concentration of 50 ng μl^{-1}. Validation results showed that ND4 primer could amplify crude species-specific DNA in samples of pigs and wild boars. In addition, this method included high precision, as shown by RSD_r and RSD_R values resulting from repeatability and reproducibility assays of less than 25%. Overall, direct qPCR method could be applied to all processed meat products and was resistant to inhibitors, except polysaccharides. Crude DNA produced from this method could be amplified with the lowest DNA concentration of 10^{-3} ng μl^{-1}. This method could be accepted as a fast and accurate method of halal detection assessments based on the results of the linearity and efficiency assays produced at 0.99 and 110%. These were similar to those of the assay acceptance threshold.

6. Conflict of Interest

The authors report no conflicts of interest.

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8. Authors Contributions

Conceptualization, JKN; methodology, JKN and MAS; software, MAS; validation, MAS, JKN and AKW; formal analysis, MAS; resources, JKN; data curation, JKN and AKW; writing and original draft preparation, MAS; writing, review and editing, AKW, ELA and KAA; supervision, JKN, AKW, ELA and KAA; project administration, MAS.

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توسعه و اعتبار سنجی روش qPCR مستقیم برای تشخیص تقلب در گوشت خوک در فرآورده های گوشتی فرآوری شده

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- بافر سلول تخریب شده،
- خوک،
- گراز وحشی

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چکیده

سابقه و هدف: تعداد مسلمانان در اندونزی در حال افزایش است؛ درنتیجه، مصرف غذاهای حلال روند افزایش دارد. این امر، تشخیص حلال بودن غذاها را نیز ضروری می‌کند. qPCR [1]مستقیم، روشی برای تشخیص حلال مبتنی بر DNA با استفاده از نمونه‌های خام DNA خام است. یکی از عواملی که بر موفقیت qPCR مستقیم تأثیر می گذارد، DNA خام است. در این مطالعه، فرآیند استخراج DNA با اصلاح مرحله تخریب سلولی به‌منظور دستیابی به DNA خام با کیفیت بالا، بهینه سازی شرایط qPCR و اعتبارسنجی آن انجام شد.

مواد و روش ها: مواد مورد استفاده برای استخراج DNA از گوشت ها و فرآورده های گوشتی شامل بافر سلول تخریب شده[2] حاوی EDTA، NaCl، SDS، NaOH و tween-20 بود. مواد مورد استفاده برای سنجش qPCR شامل DNA خام، ddH20، پرایمرها و مخلوط اصلی بود. با استفاده از بافر سلول تخریب شده با بهینه سازی شرایط تخریب سلولی (دما و زمان گرمخانه‌گذاری) ژنوم از گوشت‌ها و محصولات فرآوری شده استخراج شد. غلظت و خلوص DNA خام بررسی و سپس نتایج با روش کلروفرم: ایزوآمیل و نیز روش تجاری مقایسه شد. عصاره DNA حاصل از بافر سلول تخریب شده با استفاده از دستگاه qPCR تقویت شد. شرایط عملیات مانند تغلیظ DNA، دمای بازپخت و غلظت پرایمر بهینه شدند. با استفاده از ویژگی، تکرارپذیری[3]، تجدیدپذیری[4]، کاربردپذیری یا قابلیت اجرا[5]، استحکام و حد سنجش تشخیص، اعتبار سنجی نتایج انجام شد.

یافته‌ها و نتیجه‌گیری: دما برای تخریب سلولی بهینه در روش استخراج بافر سلول تخریب شده، ۷۵ درجه سلسیوس به مدت ۲۵ دقیقه بود. غلظت DNA از بافر سلول تخریب شده با روش کلروفرم-ایزوآمیل و روش تجاری تفاوت معنی داری نداشت (p-value = ۰/۰۹۴). شرایط بهینه برای روش qPCR، غلظت DNA ۵۰ نانوگرم میکرولیتر، دمای بازپخت ۵۳ درجه سلسیوس و غلظت پرایمر ۱۰ pmol تعیین شد. برای اعتبارسنجی نتایج، سنجش ویژگی نشان داد که این روش می‌تواند برای تشخیص گوشت خوک و گراز وحشی در گوشت‌ها با استفاده از پرایمرهای ND4 مورد استفاده قرار گیرد. علاوه بر این، بررسی تکرارپذیری شامل ۱/۰۶٪ و سنجش تجدیدپذیری شامل ۱/۵۷٪ بود. روش qPCR مستقیم را می توان برای انواع فرآورده های گوشتی فرآوری شده استفاده کرد و در برابر مهار کننده‌ها (آلژینات، یون های کلسیم، EDTA و سلولز)؛ به‌غیر از پلی ساکاریدها مقاوم است. مقدار LOD به‌دست آمده از بررسی حساسیت PCR، خطی بودن و کارایی به ترتیب ۰/۰۰۱ نانوگرم میکرولیتر، ۰/۹۹۶ و ۱۱۰ درصد بود. نتایج تأیید می‌کند که روش qPCR مستقیم برای تشخیص گوشت خوک روشی آسان، سریع، کاربردی، صحیح و دقیق است.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

۱ quantitative polymerase chain reaction or qPCR
۲ lysis buffer
۳ repeatability
۴ reproducibility
۵ applicability