

Detection and Absolute Quantification Porcine DNA in Sausages Using Quantitative Polymerase Chain Reaction (qPCR) Method

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Abstract

Identification of adulteration of processed meat products with unwanted ingredients is a crucial issue. These meat products are prone to forgery and mix with porcine. Meat source authentication is important for Muslim consumers to whom consumption of products containing pork and its derivatives in an products is prohibited. This present study aims at development of detection and quantification method of porcine DNA (*Deoxyribo Nucleic Acid*) in processed meat products, sausages. Two novel primer pairs were designed specifically targeting fragment of Displacement Loop (*D-Loop*) and cytochrome b (*cyt b*) of porcine mitochondrial DNA and to generate 139 bp and 143 bp amplicons, respectively. Detection and quantification were accomplished by Quantitative Polymerase Chain Reaction (qPCR). Porcine DNA standard curves and cycle threshold were used for quantification. The detection limit of porcine DNA was as little as 0.05 pg. Of all sausages tested (n=13), four of them contained porcine DNA as much 3.1 pg; 0.160 pg; 0.294 pg; and 0.110 pg in 0.001 mg of sausages for J, G, I, and L samples respectively. The specific qPCR assay method can be used for the detection of porcine DNA in minute amounts, which can be used for the halal authentication of food and pharmaceutical products.

Keywords

porcine DNA
D-Loop
cytochrome b
quantitative Polymerase Chain reaction (qPCR)

1. Introduction

The detection of the source of meat is a critical and important issue in food products especially in Muslim populations which are considered the food safety related to health and hygienic a halal authentication[1]. These meat products are prone to forgery and mix with porcine, it is often reported in the media about mixing it in food products labeled or sold as processed beef. One of the food subjected to adulteration target is sausages. Therefore, the development of analytical method for porcine detection is really needed[2].

There are various analytical methods that have been used to detect porcine adulteration in mixed meat samples and/or identify meat from other animal species as contaminants such as Mass Spectrometry[3], Fourier Transform Infrared Spectroscopy (FTIR)[4] and High Performance Liquid Chromatography (HPLC)[5]. These methods are specific because based on the detection of specific-specific protein of a species. A major problem

seemed to be its limitation when trying to identify the species in highly processed foods since proteins are denaturated during heat-treatments, high pressures and other processing technologies. For this reason, DNA based PCR techniques has the potential for identification of meat species. DNA has the advantage of being a relatively stable molecule, hence it is more able to withstand heat and pressure processing, and its sequence is conserved within all tissues of a species[2].

Quantitative Polymerase Chain Reaction (qPCR) is one of the development methods of PCR to detect the presence target DNA in a sample[6]. However the use of a real-time PCR approach is recommended for more accurate quantitative information and to increase specificity. The simplest, least expensive and most direct fluorescent system adapted to qPCR detection such as the SYBR Green has the advantage of being a more flexible method without the need for individual primer design[7]. In this study, mitochondrial DNA

(mtDNA) was chosen as the target for amplification. Since mtDNA has a high copy number (approximately about 100-10,000 per cell), so that it can be used for the of samples with a very limited amount of DNA. It was also has a circular shape, so mtDNA can be stable for a long time though against heat and high pressure processing. The primer targeted on the region of mitochondrial DNA is D-Loop and cytochrome b (cyt b). The two regions were used for porcine identification because in this region has high variation between organisms[8].

In this study, we will design specific primary sequences for absolute quantification of the D-Loop and cyt b of porcine mtDNA. Furthermore, the two primers were used for analysis sausages with a certain amount obtained from several supermarkets and traditional markets in Padang, Indonesia.

2. Materials and Methods

2.1 DNA Isolation

13 samples sausages were collected from local market in Padang, Indonesia and code appropriately. Samples were stored at -20°C after purchase until DNA isolation. For DNA isolation, all samples and raw meats (porcine and bovine) were grounded.

DNA isolation was carried out using PureLink™ Genomic DNA Mini Kit (Invitrogen). One hundred milligrams of samples were transferred into 2 mL Eppendorf Tube and DNA was extracted using 180 µL Lysis Buffer and 40 µL Proteinase K. This mixture was incubated at 55°C for 1 hour, followed by centrifugation. The supernatant containing DNA was pipetted into a micro tube and the added 20 µL RNase, vortex and incubated at room temperature for 2 seconds. 640 µL of the mixture containing DNA was pipetted into a spin column, centrifuged and then the liquid in the collection tube was discarded. The bound DNA is then washed twice using Wash Buffer 1 and Wash Buffer 2. The purified DNA is eluted using the Elution Buffer, incubated for 1 minute and centrifuged at maximum speed for 1.5 minutes. The concentration of total DNA and its purity was determined with the NanoDrop 2000/2000C (Thermo Scientific). Furthermore, the DNA samples were stored at -20°C until being used for analysis.

2.2 Specific Primary Design

For detection porcine DNA in sausages, the Sus Scrofa mitochondrion gen (GenBank accession number: NC_000845.1) were selected as species-specific target sequences in D-Loop and cytochrome b region using NCBI Primer-Blast software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

Design primers with melting temperature between 60-65°C that will amplify short products (100-250 bp). The most important parameter of primers for qPCR such as oligonucleotide primers are typically 15-25 nucleotides in length with 40-60% GC and avoid complementarities between the primers to avoid hairpins and primer dimers. The specificity and homology of selected primers were evaluated in bioinformatics and experimentations.

2.3 Optimization of Annealing Temperature of Primers Using Gradient PCR

Optimization of Annealing Temperature (Ta) for qPCR carried out in 10 µL reaction consisting of 1 µL DNA, 5 µL EvaGreen MasterMix, 0.5 µL each primer forward and reverse and *Nuclease Free Water* up to a volume 10 µL. The reaction was performed at 40 cycles with the condition of pre-denatured at 98°C for 2 m, denaturation at 98°C for 5 s and annealing at temperature variations (64; 63.5; 62.3; 60.4; 57.9; 56; 54.7; 54°C).

2.4 Agarose Gel Electrophoresis

Analysis of PCR amplicons was performed using agarose gel electrophoresis. Agarose gel (2.0%) was prepared by dissolving the appropriate quantities of agarose in 0.5x TBE buffer (pH 8.0) in a microwave oven. 5 µL of electrophoretic samples (DNA) were mixed with 1 µL 6X loading dye (50% glycerol, 0.08% (w/v) bromophenol blue, H₂O) and 5 µL GelRed. After electrophoresis, DNA fragments in the agarose gel were visualized and photographed using UV-transilluminator Gel Doc System (BioRad, USA).

2.5 Sensitivity of Cytochrome b (cyt b) Gene Primer of Porcine Mitochondrial DNA

Sensitivity of cytochrome b (cyt b) gene primer was determined by diluting series (5000 pg/µL, 500 pg/µL, 50 pg/µL, 5 pg/µL, 0.5 pg/µL and 0.05 pg/µL) from purified DNA. From amplification curve, we evaluated the sensitivity of the qPCR system.

2.6 Detection of Porcine DNA Using Cyt b Primer by qPCR Method

Amplification of Porcine DNA using qPCR method carried out in 10 µL reaction consisting of 1 µL DNA, 5 µL EvaGreen MasterMix, 0.5 µL each primer forward and reverse and *Nuclease Free Water* up to a volume 10

μL with condition reaction was performed at 40 cycles with the condition of pre-denatured at 98°C for 2 m, denaturation at 98°C for 5 s and annealing at optimal temperature

2.7 Making Standard Curve

A standard curve is created by diluting purified porcine DNA with known concentrations. DNA was diluted with *Nuclease Free Water* to a concentration $5,0 \times 10^{-5} \text{ ng}/\mu\text{L}$. From standard curve, will get *PCR Efficiency* (E) value, *coefficient determination* (R^2) and CV value can be determined.

2.8 Data Analysis

From the results of qPCR reaction was obtained the quantification cycle (Cq) value and starting quantity (SQ) value also known as *Copy Number Gen*, it is calculated by inputting the Cq value into the standard curve regression equation as the value of y, where the value of a is slope and b is intercept.

3. Results and Discussion

In this study detection and quantification porcine DNA in sausages using Quantitative Polymerase Chain Reaction (qPCR) to amplify porcine mitochondrial DNA in D-Loop and cyt b region. Amplification was carried out using specific primers designed in both regions for porcine DNA.

Quality of DNA Isolates

DNA template for qPCR was isolated from pork, beef and sausages. The concentration and purity of DNA were checked by measuring the absorbance (A) and determining the A260/A280 ratio using a NanoDropTM spectrophotometer as seen in Table 1. The concentration obtained from 100 mg isolation of sausage samples are between 2-26 $\text{ng}/\mu\text{L}$. While the DNA concentration of 200 mg of beef is 119, $\text{ng}/\mu\text{L}$ and pork is 112.6 $\text{ng}/\mu\text{L}$.

Pure DNA have an A260/A280 ratio in the range 1.8-2.0. Lower A260/A280 values may indicate protein contaminant, meanwhile A260/A280 values greater than 2.0 may indicate RNA contaminant. The wavelength of maximum absorption for DNA is 260nm ($\lambda_{\text{max}} = 260\text{nm}$), while the wavelength of maximum absorption for protein is 280 nm ($\lambda_{\text{max}} = 280\text{nm}$). In general, all of

DNA isolates samples are pure because A260/A280 is at 1.8-2.0 the same thing as beef and pork DNA[9].

Table 1. Concentration and Purity of DNA

No.	Sample	DNA Concentration (ng/ μL) A260 nm	Ratio A260/280 nm
1	Sample A (100 mg)	4.675	1.91
2	Sample B (100 mg)	2.672	1.86
3	Sample C (100 mg)	19.583	1.95
4	Sample D (100 mg)	11.115	1.92
5	Sample E (100 mg)	4.080	1.78
6	Sample F (100 mg)	26.008	1.89
7	Sample G (100 mg)	11.819	1.86
8	Sample H (100 mg)	8.393	1.76
9	Sample I (100 mg)	2.689	1.95
10	Sample J (100 mg)	2.175	2.07
11	Sample K (100 mg)	8.728	1.90
12	Sample L (100 mg)	6.731	1.93
13	Sample M (100 mg)	7.383	1.91
14	Beef (200 mg)	119.9	2.07
15	Pork (200 mg)	112.6	2.01

Primer Specificity Analysis for D-Loop and Cyt b Porcine Mitochondrial DNA

Primer specificity were designed in D-Loop and cytochrome b (cyt b) region in porcine mitochondrial DNA using Primer-Blast software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

Porcine mitochondrial DNA (*Sus Scrofa*) with an access number NC_000845.1 used as a template DNA to design primers. Primers were designed must be accordance with criteria for amplification with qPCR. Design primers with melting temperature between 60-65°C that will amplify short products (100-250 bp). The most important parameter of primers for qPCR such as oligonucleotide primers are typically 15-25 nucleotides in length with 40-60% GC and avoid complementarities between the primers to avoid hairpins and primer dimers.^[10] Based on these criteria, primer specificity were selected to amplify pork DNA in D-Loop and cyt b regions as seen in Table 2

Table 2. Sequence of primers at target region D-Loop and cyt b region of mitochondrial DNA

Primer	Sequen (5'->3')	Length	Amplicon	T _m (°C)	GC%
F_DLoopScrofa	CCTTGCCAAACCCCAAAAACA	21 nt	139 bp	60,7	47,62
R_DLoopScrofa	GGGTAAGTGCCTGCTTTCGTA	21 nt		60,34	52,38
F_CytbScrofa	CCCTTATATCGGAACAGACCTG	23 nt	143 bp	60,06	52,17
R_CytbScrofa	GCAGGAATAGGAGATGTACGGT	23 nt		61,87	52,17

Primers amplified in the D-Loop region are given the name DLoopScrofa and primers in the cytochrome b region are named CytbScrofa. Primers specificity have been designed impacts the entire DNA amplification process, primers were analyzed using Primer-Blast software and also experiments. Using a bioinformatics analysis, primer DLoopScrofa and CytbScrofa were tested on all species in NCBI so it can be known the primers were proven precise only amplify the target fragment (mitochondrial DNA porcine). The test results showed the designed primers can only specifically amplify porcine.

Experimentally, DLoopScrofa and CytbScrofa primers were tested on bovine and porcine DNA using conventional PCR. The agarose gel images of PCR products, obtained from conventional PCR reactions using two species-specific primers for porcine detection in pork and beef meat. PCR amplification for porcine DNA yielded a band of 139 bp using DLoopScrofa primers and a band of 143 bp using CytbScrofa primers. Both specific primers amplify porcine DNA while bovine DNA is not amplified as seen in Fig.1.

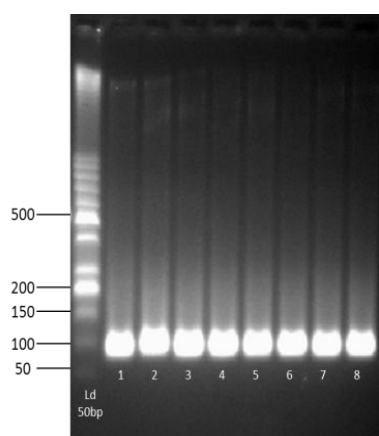


Fig 1. Agarose gel electrophoresis of PCR products for specificity test. CytbScrofa and DLoopScrofa primer for porcine DNA (1 and 3) and bovine (2 and 4). Both

specific primers amplify porcine DNA while bovine DNA is not amplified.

CytbScrofa primers has a band length of 143 bp and DLoopScrofa has a band length of 139 bp. Both primers were proven to not produce cross-amplification with bovine DNA. It was proven that both primers only amplified porcine DNA while bovine DNA is not amplified. Further, optimization of qPCR condition for this study only uses CytbScrofa primers.

CytbScrofa Primers Annealing Temperature

Optimization methods were performed to determine the optimum annealing temperature (Ta). A total of 8 positive control samples containing porcine DNA were used for Ta optimization with various Ta ranged from 54°C to 64°C both for DLoopScrofa primers and for CytbScrofa. The effects of temperature variations to find the optimum annealing temperature produced amplification curves and melting curve (Fig. 2). The amplification curve is a visualization of the fluorescent signal that has been formed since the amplification process began. Fluorescent (SYBR-Green) which intercalates into double-stranded DNA as it accumulates during the PCR reaction thereby producing a fluorescent signal that can be quantified. The amplification curves showed typical four phases of amplification including baseline, exponential phase, linear phase and a plateau[7].

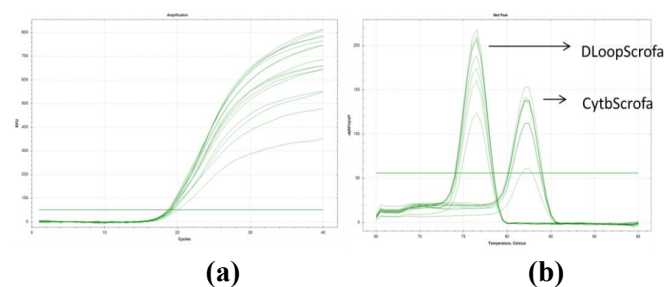


Fig 2. Curve optimization annealing temperature DLoopScrofa and CytbScrofa primers (a) Curve amplification and (b) Melt Peak optimization annealing temperature.

Based on the results of the optimization Ta amplification curves, it seemed that fluorescent (the number of amplicons) increases with increasing cycles in reaction. The exponential phase of amplification curve is an important factors determine the threshold to determine the value of Cq. From the curve, it seemed all variations of temperature have almost the same Cq values. Fig.2 (b) is a melt peak that DLoopScrofa and CytbScrofa primers were showed specific results, both primers have the same melting temperature (Tm) value marked by the formation of peaks at the same temperature.

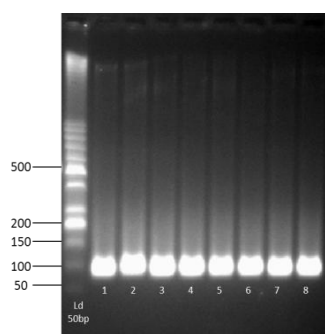


Fig 3. Visualization of electrophoresis gradient Ta CytbScrofa primers PCR products. Band position (1-8) was PCR products (143 bp) resulting from annealing temperature 54.0; 54.7; 56.0; 57.9; 60.4; 62.3; 63.5 and 64°C.

Same results was shown by electroforegram, PCR products using CytbScrofa primers were obtained from all variations of annealing temperature as seen in Fig 3. All variations were showed the same quality products PCR, so the choice of temperature is quite varied. In theory, the best annealing temperature (Ta) is around $\pm 5^{\circ}\text{C}$ from the melting temperature (Tm). CytbScrofa primers has melting point 60.0°C and 61.87°C , consider this fact the optimum annealing temperature should be

at $64.0\text{--}65.0^{\circ}\text{C}$. Therefore, the annealing temperature (Ta) used for this study was 64.0°C [10].

Sensitivity of CytbScrofa Primers

The sensitivity of CytbScrofa primers using qPCR method was conducted to determine the smallest DNA concentration that can be amplified and visualized on the amplification curve of the qPCR results[11].

Sensitivity determination was performed with various porcine DNA concentration in reaction : 5000 pg/ μL , 500 pg/ μL , 50 pg/ μL , 5 pg/ μL , 0.5 pg/ μL and 0.05 pg/ μL (Fig. 4). The smallest DNA concentration that can be amplified and detected is 0.05 pg/ μL .

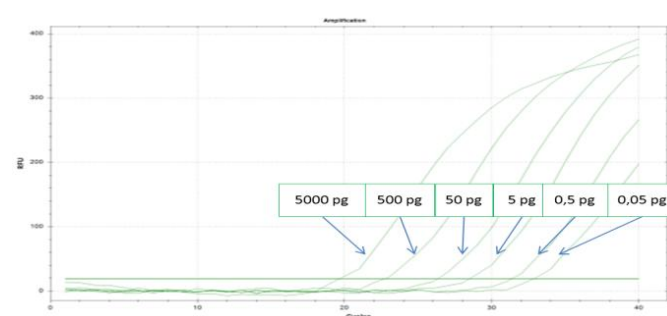


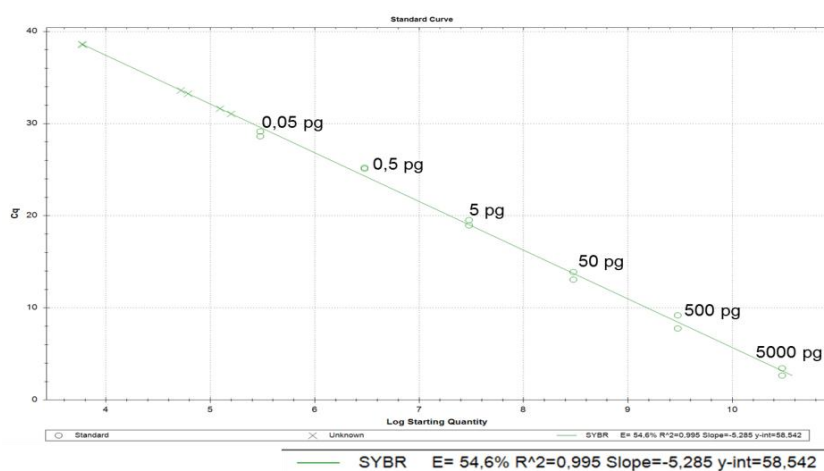
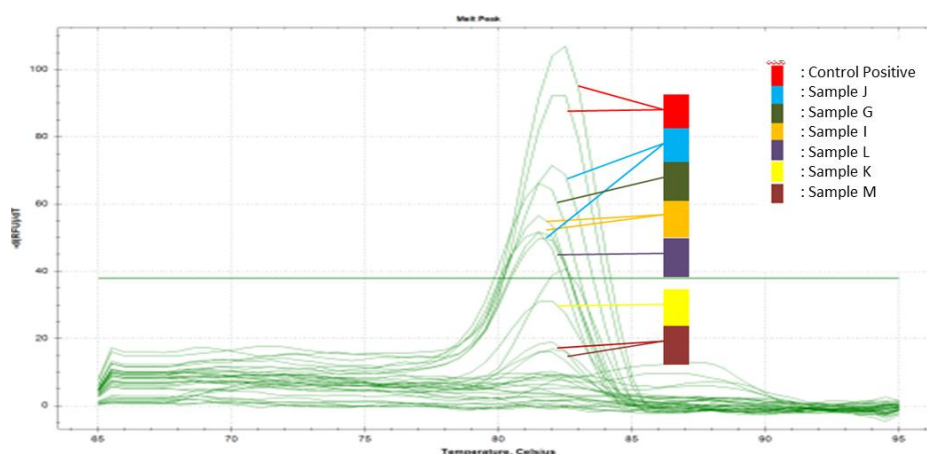
Fig 4. Visualization of amplification curve for sensitivity test primer CytbScrofa (5000 pg; 500 pg; 50 pg; 5 pg; 0.5 pg; and 0.05 pg). The lowest amount of DNA that could be amplified is 0.05 pg.

Standard Curve of Porcine DNA

A standard curve (plot of Cq values/crossing point of different standard dilutions against log of amount of standard) is generated using a dilution series at 6 different concentrations of the standard (Table. 3). The Cq values of the standard samples are determined. Then, the Cq value of the unknown sample is compared with standard curve to determine the amount of target in the unknown sample (Fig. 5)[12].

Table 3. Quantification Standard Data Analysis of Amplification using CytbScrofa Primers

Sample	Cq Mean	Starting Quantity (SQ)	Log SQ	Amplicons Mass 143 bp (ng)	DNA Mass (pg)
Standard 6	3.05	3.00×10^{10}	10.477	1.567×10^{-10}	5000
Standard 5	8.48	3.00×10^9	9.477	1.567×10^{-10}	500
Standard 4	13.48	3.00×10^8	8.477	1.567×10^{-10}	50
Standard 3	19.24	3.00×10^7	7.477	1.567×10^{-10}	5
Standard 2	25.16	3.00×10^6	6.477	1.567×10^{-10}	0.5
Standard 1	28.88	3.00×10^5	5.477	1.567×10^{-10}	0.05

**Fig 5.** Standard Curve of porcine DNA amplification using CytbScrofa primers. The criteria of this standard curve are $R^2=0.995$; $E= 54.6\%$ dan $CV=4.29\%$ **Fig 6.** Visualization of amplification curve for DNA of porcine, bovine and 13 samples (A; B; C; D; E; F; G; H; I; J; K; L; and M). Control positive and four samples was positive contain porcine DNA.

Amplification CytbScrofa primers using qPCR method was expressed as detection limit, porcine DNA can still be amplified up to 0.05 pg. Therefore, it can be judged that LoD value of DNA to be amplified is 0.05 pg. The coefficient determination (R^2) obtained for the relationship between log of DNA concentration (axis) and cycle threshold (Cq) was 0.995, with y-intercept of 58.542 and amplification efficiency (E) is 54,6%. (Fig. 5). The coefficient of variation (CV) on repeatability analysis was 4,295. The ideal value of standard curve for absolute quantification are $R^2 \geq 0.900$, $E= 90-110\%$,

and $CV \leq 25\%$. Hence, the standard curve is less feasible for absolute quantification because only two out of three ideal value that meets the criteria.

In other study using qPCR method as determination of porcine contamination on dendeng have ideal value standard curve for absolute quantification with coefficient determination (R^2) value was 0.983, amplification efficiency (E) is 102.4% and coefficient of variation (CV) value of 17.2% using cyt b primer[13]. Also, pork detection in commercial food products have same value standard curve with R^2 value

of 0.992, amplification efficiency (E) is 92% and coefficient of variation (CV) value of 3.3% using cyt b primer[14].

Detection and Quantification Porcine DNA in Sausages

Amplification DNA of porcine, bovine and samples of sausages was identified using new specific primer for CytbScrofa primer in mitochondrial DNA sequence of porcine. Porcine DNA was used as a positif control and bovine DNA was used as negative control in PCR reaction. The developed assay was further applied to the thirteen commercial sausages products for identification purposes (Fig. 5).

Analysis the results of porcine DNA amplification and sausages samples was seen in the amplification curve in Fig. 5. Identify porcine DNA contaminants using the qPCR method, obtained the results that among all samples analyzed found four samples which was positive contain porcine DNA. The four samples were J, G, I and L. Mitochondrial DNA from porcine and four samples has the same Tm value of 82.5°C. Peak of amplification showed that SYBR Green molecules are detected have formed intercalate with PCR product[15].

Cq values are inverse to the amount of target nucleic acid that is in sample, and correlate to the number of

target copies in the sample. Lower Cq values (typically below 29 cycles) indicate high amounts of target sequence, while higher Cq values mean lower amounts of target nucleic acid (Table 4). From Table 3. can be seen that the highest standard Cq value of 28.88 is at the smallest concentration standard (standard 1). This Cq value is defined as the Cq LoD value. So that, the Cq value of the sample which is below the Cq LoD value was stated to contain positive porcine DNA. The sample Cq value that is above Cq LoD value is stated as false positive, that is a positive detected sample that actually does not exist[16].

Based on the LoD values on the PCR reaction, the test was also done using specific primers with the qPCR method of 13 sausages samples from local markets in Padang (sample A-M). Based on Fig. 6, the positive control showed a rise above threshold line with Cq value 27.64. Of all sausages tested, four of them contained porcine DNA as much 3.1 pg; 0.160 pg; 0.294 pg; and 0.110 pg in 0.001 mg of sausages for J, G, I, and L samples respectively. Porcine DNA was not detected in all of samples as seen in Table 4. Several methods of halal detection or mixing of porcine DNA in processed meat products detect the presence of 1 pg of pork DNA in 1 µL of processed food products and 1 mg in 100 mg of meatballs while in dendeng and corned beef products show negative results[13,17,18].

Table 4. Detection and quantification absolute data of porcine DNA in sausages

Sample	Cq Mean	Starting Quantity (SQ)	Log SQ	Amplicons Mass 143 bp (ng)	DNA Mass (pg)
A	30.71	1.85×10^5	5.266	1.567×10^{-10}	2.89×10^{-2}
B	31.64	1.23×10^5	5.090	1.567×10^{-10}	1.93×10^{-2}
C	31.35	1.24×10^5	5.093	1.567×10^{-10}	1.94×10^{-2}
D	30.42	2.09×10^5	5.322	1.567×10^{-10}	3.29×10^{-2}
E	33.43	6.12×10^4	4.786	1.567×10^{-10}	9.59×10^{-3}
F	38.60	6.00×10^3	3.774	1.567×10^{-10}	9.32×10^{-4}
G	25.76	1.59×10^6	6.024	1.567×10^{-10}	2.49×10^{-1}
H	36.47	1.50×10^4	4.176	1.567×10^{-10}	2.35×10^{-3}
I	21.51	1.02×10^7	7.008	1.567×10^{-10}	1.60×10^{-1}
J	14.70	1.97×10^8	8.296	1.567×10^{-10}	3.10
K	30.03	2.48×10^5	5.395	1.567×10^{-10}	3.89×10^{-2}
L	27.64	7.03×10^6	5.847	1.567×10^{-10}	1.10×10^{-1}
M	30.41	2.10×10^5	5.322	1.567×10^{-10}	3.29×10^{-2}

4. Conclusions

Based on this study, it can be concluded that DLoopScrofa and CytbScrofa primers in mitochondrial region was been designed specific for absolute quantification with the lowest concentration that could be amplified and detected is 0.05 pg. Of all sausages tested, four of them contained porcine DNA as much 3.1 pg; 0.160 pg; 0.294 pg; and 0.110 pg in 0.001 mg of sausages for J, G, I, and L samples respectively. The specific qPCR assay method can be used for the detection of porcine DNA in minute amounts, which can be used for the halal authentication of food and pharmaceutical products.

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Formal Analysis: S., S., N..

Funding acquisition: S

Investigation: S., N.

Methodology: S

Project administration: S., N..

Visualization: S., S., N.

Writing – review & editing: S., S., N.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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