

Validation of a Non-Specific Dye Real-Time PCR Assay for Porcine Adulteration in Meatball Using ND5 Primer

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ABSTRACT

Porcine adulteration in meatball samples were analyzed using real-time polymerase chain reaction (RT-PCR), based on the ND5 primer obtained by previous study. This work consisted of three stages which were annealing temperature optimization, method validation, and application. DNA template was extracted using phenol-CIAA (chloroform-iso amyl alcohol) method. The optimum annealing temperature for ND5 primers (forward primer 5'-CATTGCCTCACTCACATTAACC-3' and reverse primer 5'-AAGAGAGAGTTCTACGGTCTGTAG-3') was 58.0 °C, obtained after testing annealing at 50.5 to 59.5 °C gradient temperature with 5 °C interval. Melting curve analysis was done at 65.0 to 95.0 °C, with increasing temperature for 0.5 °C per 2 sec. Method was validated for its specificity, precision and limit of detection. RT-PCR method with ND5 primers produced 227 bp DNA fragment with 78.50 °C T_m value. From eight commercial meatball samples, one was detected containing porcine. The methods showed high specificity and precision, with experimentally determined limits for porcine were no less than 1%.

Keywords: meatballs; porcine DNA; method validation; ND5 primer; real-time PCR

ABSTRAK

Telah dilakukan analisis kontaminasi babi pada bakso dengan real time polymerase chain reaction (RT-PCR) berdasarkan perbedaan urutan DNA mitokondria. Penelitian yang dilakukan meliputi 3 tahapan yakni optimasi, validasi, dan aplikasi metode, diawali dengan isolasi DNA menggunakan fenol-KIAA (kloroform-iso amil alkohol). Optimasi metode RT-PCR dilakukan dengan menguji gradien temperatur (50,5–59,5 °C) untuk memperoleh temperatur annealing (T_a) optimal dari primer ND5 forward (5'-CATTGCCTCACTCACATTAACC-3') dan primer ND5 reverse (5'-AAGAGAGAGTTCTACGGTCTGTAG-3'). Kondisi PCR yang digunakan berupa pre-denaturasi pada temperatur 95 °C selama 30 detik, denaturasi pada temperatur 95 °C selama 2 detik dan annealing pada T_a optimal selama 5 detik serta melting curve analysis pada temperatur 65,0–95,0 °C. Metode divalidasi dengan menentukan spesifisitas, presisi dan batas deteksi. Aplikasi metode dilakukan dengan menguji sampel produk bakso di pasaran. Hasil RT-PCR dengan primer ND5 berupa fragmen DNA dengan T_m (titik leleh) sebesar 78,5 °C dengan ukuran fragmen 227 pb. T_a optimal untuk PCR adalah 58,0 °C. Metode memiliki spesifisitas dan presisitas yang tinggi dimana primer ND5 hanya mengamplifikasi DNA babi dan tidak terbentuk ampikon pada kontrol negatif serta hasil yang konsisten setelah 10 kali pengulangan pada waktu isolasi yang berbeda untuk masing-masing kontrol positif dan negatif. Metode masih dapat mendeteksi sampai dengan 1% kontaminasi babi. Analisis RT-PCR terhadap 8 sampel bakso berhasil mendeteksi 1 sampel dari pasar tradisional yang positif terkontaminasi babi.

Kata Kunci: bakso; DNA babi; validasi metode; primer ND5; real-time PCR

INTRODUCTION

The increased awareness of consumers regarding the composition of foods has resulted in the need to verify the labelling statements. The incorrect labelling of foods represents a commercial fraud, considering the consumer acquisition. It is very important to establish that species of high commercial value are not substituted, partial or entirely, by other lower value species [1].

The information given to consumers is also essential for them to choose certain foods over others [2]. These practices are of concern for not only economic reason since it leads to unfair competition among producers, but also ethics health and religious since the consumption of certain species is not allowed in some religions [3]. Thus, when considering all the referred concerns, the need to verify the labelling statements is a crucial aspect of consumers, food industries, and food authorities [4].

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Meatball, or known as 'bakso' in Indonesia, is processed comminuted meat which can be classified as restructured meat and is very popular throughout the world. They can be formulated using beef, chicken, porcine, and fish muscle. However, the beef meatball is very popular and widely found in markets [5]. Substitution of beef in meatball formulations with lower valued meats such as porcine frequently takes place to coup up with market competition and also to earn economic gain [6].

The need for accurate and reliable methods for species identification in foods has increased steadily in the past few years [7]. Recently, a range of analytical approaches has been developed broadly based on detecting either protein or DNA molecules. Among the first, immunoassays are the most widely used techniques. The main advantages of the applied immunoassays are the good sensitivity along with a capability of a high sample throughput. 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 [8]. For this reason, immunological analyses have been replaced by DNA-based methods. 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 an individual [9].

The PCR amplification is based on the hybridization of specific oligonucleotides (primers) and synthesis, *in vitro*, of millions of copies flanked by those primers [10]. Conventional PCR has been succeeded to detect porcine contamination in meatball [11]. 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 real-time PCR detection such as the SYBR Green has the advantage of being a more flexible method without the need for individual probe design. To verify the specificity of amplified products, DNA melting curves can distinguish false positive signals due to non-specific amplification or primer-dimers [12]. Real-time PCR based assays have previously been reported by some researcher for the identification of animal-derived material in meat mixtures by amplifying mtDNA genes as 12 S rRNA [15], cytochrome b gene [3,16-19,], ND2, ND5 and ATP 6-8 [20], and 18S rRNA [3].

From the above discussion, it is concluded that a reliable method is necessary for the precise results for the accurate identification of fraudulent practices in meat control systems. In this work, an SYBR green real-time PCR approach is proposed as a simple, fast, sensitive

and reliable method for porcine meat detection. The method was validated and subsequently applied to detecting the presence of porcine meat in meatball products available commercially.

EXPERIMENTAL SECTION

Materials

Materials used in this study were: ingredients needed to make meatball such as porcine meat, tapioca, eggs, salt, pepper and garlic, all from Yogyakarta local market. Commercially meatball sample was collected from the supermarket and local market (halal-labeled and non-labeled). Chemicals used were Tris base (Sigma-Aldrich), ethidium bromide (Sigma-Aldrich), hydrochloric acid (Merck), ethylenediaminetetraacetic acid (Merck), sodium dodecyl sulphate (Merck), sodium chloride (Merck), phenol (Merck), chloroform (Merck), isoamyl alcohol (Merck), 2-propanol (Merck), ethanol absolute (Merck), proteinase-K (Invitrogen), DNA marker 100 bp (Invitrogen), SsoFast EvaGreen™ Supermix (Bio-Rad), agarose (Spain), loading buffer (Vivantis), primer forward ND5: 5'-CAT TCG CCT CAC TCA CAT TAA CC-3' and primer reverse ND5: 5'-AAG AGA GAG TTC TAC GGT CTG TAG-3' (1st BASE), sterile aquabidest (LPPT-UGM), DNase and RNase free water (Gibco™).

Instrumentation

Instruments used in this study were: RT-PCR CFX96 (Bio-Rad), spectrophotometer UV-1700 (1024E) 230VCE (Shimadzu), Sorvall Legend Micro 17R centrifugation (Thermo Scientific), electrophoresis [Bio-Rad (Wide minisub[p] cell GT)], digital balance (Denver AA-250), micropipettes (Gilson), UV light (Bio-Rad), water bath (OSK Seiwa Reiko), vortex (Termplyne), Incubator EM-200T (Sakura), microwave (Sanyo), and digital camera (Kodak easysshare Z650).

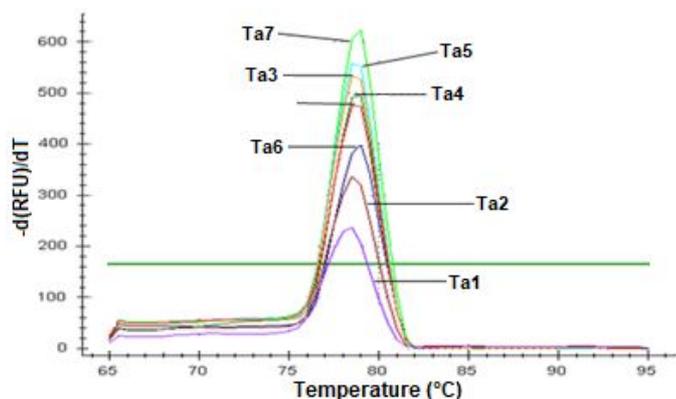
Procedure

Samples preparation

Reference meatball sample was prepared in the laboratory with cattle and porcine meat. Samples were minced separately, and the reference binary mixtures containing 0%, 1%, 10%, and 100% (w/w) of porcine in cattle meat were prepared to a final weight of 100 g (Table 1). The reference meatball sample and commercial samples were immediately stored at -20 °C.

Table 1. Reference meatball sample used for this study

Sample	Porcine meat (g)	Cattle meat (g)	Total weight (g)
Porcine meatball 0%	0	100	100
Porcine meatball 1%	1	99	100
Porcine meatball 10%	10	90	100
Porcine meatball 100%	100	0	100

**Fig 1.** Melting curve analysis for Ta optimization

DNA extraction

DNA was extracted using the phenol-CIAA (chloroform-iso amyl alcohol) method. Briefly, 200 mg of ground sample was mixed with 2 mL buffer lysis. The mixture was then transferred to a microtube, followed by addition of 30 μ L proteinase-K. After incubation in water bath at 37 $^{\circ}$ C for 24 h, the suspension was vortexed for 2 min and transferred into a new microtube. Phenol-CIAA was added to the suspension with the same volume (1:1) and mixed by shaker at 80 rpm for 30 min. Separation was performed by centrifuge (5 min, 12000 rpm, 4 $^{\circ}$ C) and supernatant was mixed with phenol-CIAA with the same volume as supernatant (1:1) for 5 min. The mixture was centrifuged again (5 min, 12000 rpm, 4 $^{\circ}$ C) and 500 μ L of supernatant was precipitated with the addition of 500 μ L 2-propanol (1:1) followed by incubation at -20 $^{\circ}$ C for 30 min. Extracted DNA was separated by centrifugation (5 min, 12000 rpm, 4 $^{\circ}$ C), the pellet was collected and washed with 500 μ L ethanol 70% and centrifugation (5 min, 12000 rpm, 4 $^{\circ}$ C). After disposing the ethanol and drying the extracted DNA, extracted DNA was mixed with 30 μ L tris-EDTA to a new microtube. This suspension was stored at -20 $^{\circ}$ C for further use. The quality of extracted DNA was analyzed by electrophoresis on a 1% agarose gel in TBE buffer 0.5x for 30 min at 50 V. The agarose gel was visualized under UV light, and a digital image was obtained using a digital camera.

DNA quantification

Extracted DNA was quantified by spectrophotometer UV-Vis. The DNA concentration was determined by absorbance at 260 nm (1 absorbance unit

correspond to 50 ng/ μ L of dsDNA) [21]. The purity of the extract was evaluated based on the ratio of the absorbance at 260 and 280 nm, and values between 1.7 and 2.0 were obtained [22].

Real-time PCR optimization

Annealing Temperature (Ta) Optimization for real-time PCR was carried out in 20 μ L containing 10 μ L SsoFast EvaGreenTM supermix, 1 μ L primer forward ND5, 1 μ L primer reverse ND5, 50 ng DNA sample (reference meatball sample containing porcine meat) and DNase and RNase free water. The real-time PCR assays were performed using following condition: 95 $^{\circ}$ C for 30 sec (predenaturation), 35 cycles of denaturation at 95 $^{\circ}$ C for 2 sec and annealing at various temperature (50.5; 51.1; 52.3; 54.0; 56.2; 58.0; 59.0; and 59.5 $^{\circ}$ C). For melting curve analysis, the temperature was increased by 0.5 $^{\circ}$ C per 2 sec from 65.0 to 95.0 $^{\circ}$ C.

Detection of porcine meat using real-time PCR

Amplification by real-time PCR were carried out in 20 μ L total reaction volume containing 10 μ L SsoFast EvaGreenTM supermix, 1 μ L primer forward ND5, 1 μ L primer reverse ND5, 50 ng DNA sample and DNase and RNase free water using following condition: 95 $^{\circ}$ C for 30 sec (predenaturation), 35 cycles of denaturation at 95 $^{\circ}$ C for 2 sec and annealing at optimal temperature. For melting curve analysis, the temperature was increased by 0.5 $^{\circ}$ C/2 sec from 65.0 to 95.0 $^{\circ}$ C. Amplified fragments were analyzed using electrophoresis on a 2.0% agarose gel with the addition of 4 μ L ethidium bromide in TBE buffer for 30 min at 100 V. The result was visualized under UV light, and a digital image was obtained using a digital camera.

Validation method of real-time PCR

Specificity test. This test was begun with extraction of positive control (porcine meatball 100%) and negative control (porcine meatball 0%). Extracted DNA were quantified by spectrophotometer UV-Vis at 260 nm and 280 nm. Amplification by real-time PCR were carried out in 20 μ L total reaction volume containing 10 μ L SsoFast EvaGreenTM supermix, 1 μ L primer forward ND5, 1 μ L primer reverse ND5, 50 ng DNA sample and DNase and RNase free water using following condition: 95 $^{\circ}$ C for 30 sec (predenaturation), 35 cycles of denaturation at 95 $^{\circ}$ C for 2 sec and annealing at optimal temperature. For melting curve analysis, the

temperature was increased by 0.5 °C/2 sec from 65.0 to 95.0 °C. The amplified fragments were analyzed by electrophoresis in a 2.0% agarose gel with addition of 4 µL ethidium bromide in TBE buffer for 30 min at 100 V. Agarose gel was visualized under UV light and a digital image was obtained using a digital camera.

Precision test. Precision test was performed by repetition of detection method for 10 positive controls and 10 negative controls. Every repetition held in different time for contamination prevention.

Limit of detection determination. Limit of detection (LOD) determination was performed with various porcine contamination in meatballs: 0%, 1%, and 10% (w/w%). Each was treated with the same detection method in the same time.

Application. The validated method was applied to the various commercial meatball samples. Eight meatball samples were picked randomly, 4 samples from the supermarket (halal labeled), and 4 samples from traditional market (non-labeled).

RESULT AND DISCUSSION

Real-time PCR Optimization

The ND5 mitochondrial gene was chosen as a target for porcine DNA detection because PCR assays based on mitochondrial genes show improved sensitivity when compared to single or low copy nuclear DNA targets. The primers used in this study are primer forward ND5: 5'-CAT TCG CCT CAC TCA CAT TAA CC-3' and primer reverse ND5: 5'-AAG AGA GAG TTC TAC GGT CTG TAG-3' obtained from previous study by Kesmen et al. [23].

Optimization methods were performed to determine the optimum annealing temperature (T_a). A total of 8 positive control samples containing porcine DNA (porcine meatball 100%) were used for T_a optimization with various T_a ranged from 50.5 to 59.5 °C. Based on the results of the optimization T_a amplification curves (Table 2), the overall positive controls that were tested yielded varies cycle number threshold (C_t) between 17.71 to 19.62 as presented in Table 2. Highest relative fluorescence units (RFU) were shown for T_a 51.1 °C. ND5 mitochondrial DNA primers has melting point at 60.6 °C and 61.0 °C for the forward and reverse primer, respectively. Consider this fact, the optimum annealing temperature should be about 55.6 °C. Therefore, instead of T_a 51.1 °C, the T_a used for this study was 58.0 °C, because it produced low C_t , high RFU and could prevent primer mispriming due to low T_a (Table 2).

Further analysis were done by heating the PCR amplicon at the end of the reaction by raising the temperature from 65.0 °C to 95.0 °C to melt the double-stranded DNA into a single strand, and the difference in

Table 2. C_t and RFU value for various annealing temperature

T_a (°C)	Code	C_t	RFU
59.5	Ta ₁	19.62	743
59.0	Ta ₂	18.74	1,220
58.0	Ta ₃	17.71	2,077
56.2	Ta ₄	18.26	1,847
54.0	Ta ₅	17.88	2,262
52.3	Ta ₆	18.28	1,835
51.1	Ta ₇	17.85	2,399
50.5	Ta ₈	18.00	1,720

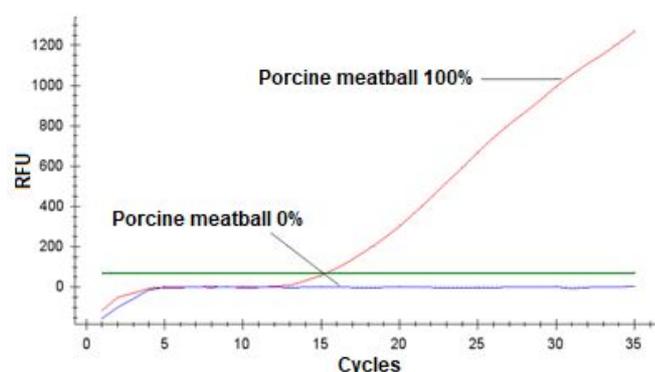


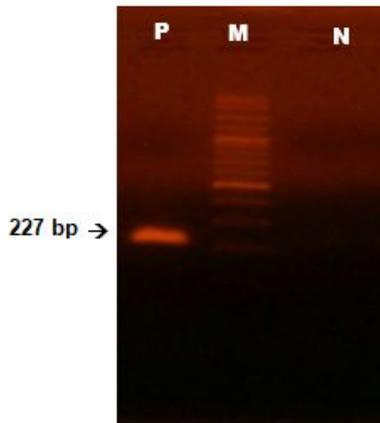
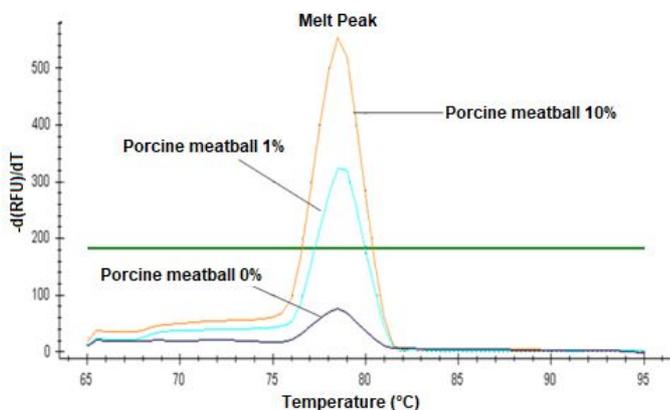
Fig 2. Amplification pattern for specificity test: positive control (red line), negative control (blue line) and threshold (green line)

the fluorescence signal was measured. At the melting point, the two strands of DNA amplification product were separated and intensity of fluorescence decreased significantly. Melting curve analysis (MCA) describes the rate of change of the RFU as the first negative derivatization of fluorescent signal versus time divided by temperature (T) ($d(\text{RFU})/dT$). Positive controls containing porcine DNA with different T_a showed similar curves which rose above the threshold 182.51 with melting temperature (T_m) at 78.5 °C and 79.0 °C (Fig. 2). T_m is the temperature at which 50% of the DNA amplicon is in a double-stranded configuration. The T_m depends on various factors including the amplicon length and the nucleotide sequence, which is specific for each amplicon [24]. From Kesmen et al. [25], 78.5 °C is known as the T_m of PCR products of ND5 primers. With this method, a sample was declared containing porcine DNA when the analysis in the porcine specific RT-PCR system with ND5 primer produced an amplification product with melting temperature 78.5 °C, corresponds to sequence-specific hybridization between the primers and the homologous porcine DNA sequence.

Qualitative analysis of RT-PCR were performed using agarose gel electrophoresis. DNA amplicons size for the optimization of T_a were approximately 200 bp. Amplicon size of the primer ND5 itself based on a study by Kesmen et al. [25] is 227 bp, corresponded to the

Table 3. RT-PCR result for sample C1-C4

Sample	Code	Ct	RFU	Melting point (°C)	Peak height
Traditional market meatball 1	C ₁	-	-17.1	-	-
Traditional market meatball 2	C ₂	34.94	21.1	-	-
Traditional market meatball 3	C ₃	-	-8.95	-	-
Traditional market meatball 4	C ₄	25.35	750	78.5	353.45
Porcine meatball 100%	+	16.19	2,037	78.5	589.45
Porcine meatball 0%	-	-	-6.79	-	-

**Fig 3.** Specificity test electroforegram: positive control (P), DNA marker 100 bp (M), and negative control (N)**Fig 4.** Melting curve analysis for LOD determination: porcine meatball 10% (orange line), porcine meatball 1% (blue line), porcine meatball 0% (purple line) and threshold (green line)

specific amplicon produced by amplification with ND5 primers.

Assay Validation

Specificity test

Assay specificity of developed RT-PCR was confirmed when amplification in the sample containing no porcine in the same matrix was not detected. To demonstrate specificity, positive (100% porcine meat)

and negative control (100% cattle meat) were compared in this test.

From Fig. 2, positive control showed the increasing amount of the RFU above the threshold line, indicating the occurrence of the amplification process, while negative control produced no amplification. The positive control showed a peak above the threshold 106.10 for MCA, had T_m value 78.5 °C and produced a 227 bp band on electroforegram (Fig. 3), while the negative control did not produce any peak for MCA and did not produce any band on electroforegram (Fig. 3).

Precision test

The precision test was aimed to evaluate the influence of analysis time with the result. All of the detection methods were done for ten positive controls and ten negative controls at different time. All of the positive controls extracted at different times showed a uniform curve above the 51.14 threshold and give Ct values varying between 13.63 to 23.52 with a total value of amplicon between 456 to 2309. The standard deviation (SD) of the positive controls precision test was 2.87 with 18.07% relative standard deviation (RSD). The RSD value meet the criteria for precision of RT-PCR method which should not exceed 25% [26] Further analysis by MCA gave the same result with melting point at 78.5 °C. All of the positive controls showed amplification, although the peak heights were varying between 289.73 to 709.92. According to the analysis of DNA fragments by electrophoresis, all of the amplicons for positive controls also showed uniform clear 227 bp band.

For the negative controls, RFU showed a line below the threshold 139.13, therefore the negative controls did not have a Ct value with a relatively low RFU. Same result also given from the MCA and electrophoresis analysis, which all of the negative controls did not produce any peak and any band. This result implicated that this method has a great precision.

LOD determination

Limit of detection (LOD) determination was performed with various porcine contamination in meatball: 0%, 1%, and 10% (w/w%). Each treated with the same detection method at the same time. Amplification result demonstrated that 1% and 10%

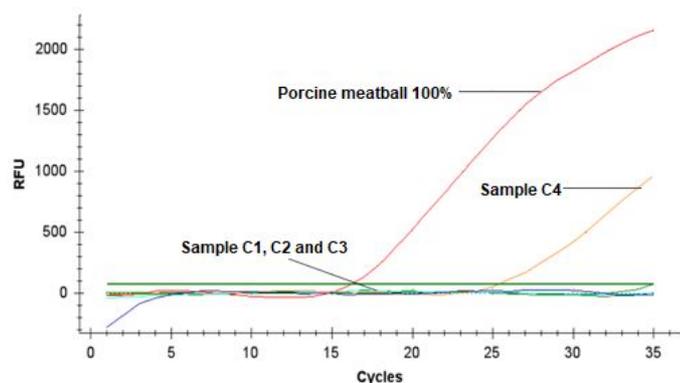


Fig 5. Amplification pattern for samples purchased from traditional market

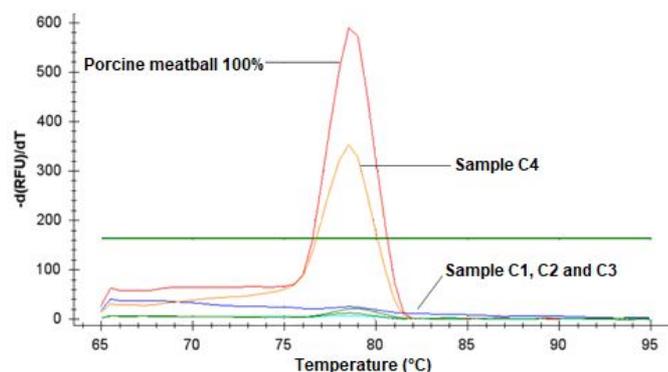


Fig 6. Melting curve analysis for meatball samples purchased from traditional market

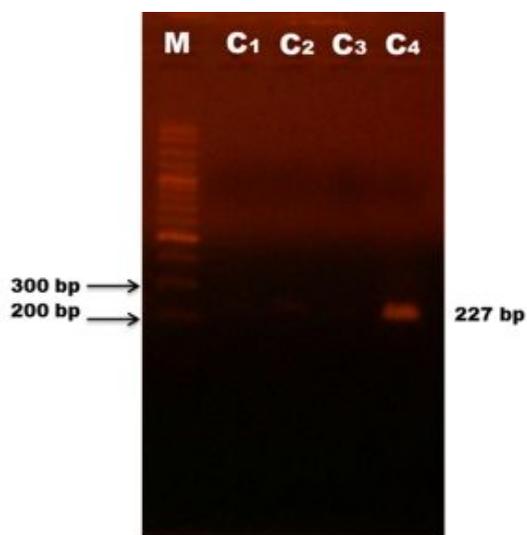


Fig 7. Electrophoresis: DNA marker 100 bp (M), meatball samples from traditional market (C1-C4)

porcine meatballs were all amplified with Ct value 25.48 and 21.52, respectively. MCA also showed peaks above the threshold 183.35 with the T_m value of 78.5 °C, with peak height 324.11 for 1% porcine meatball and 554.34 for 10% porcine meatball (Fig. 4). Same result was shown by electrophoresis, in which 1% porcine meatball and 10% porcine meatball produced a 227 bp band, while negative control did not produce any. According to these results, the RT-PCR with ND5 primers method proved to be a sensitive method, which could detect no less than 1% porcine contamination.

Detection of porcine meat using real-time PCR

The optimized PCR conditions were subsequently applied to the commercial samples to detectify the presence of porcine meat and the compliance with label statements. Analysis of the samples meatballs from supermarket brand A (SA), brand B (SB), brand C (SC) and brand D (SD) were detected by RT-PCR

amplification showed no increase in the curve with a low Ct value and RFU intensity, while the positive control showed a rise of the curve above the threshold 67.46 with Ct value 24.12 and RFU 1122. For MCA, no peak was produced by all four. These results indicated that no amplification was achieved with all four samples. This result was also confirmed by electrophoresis analysis. Compared with DNA marker 100 bp, all of the DNA samples did not show any band. Therefore, all supermarket brand samples were not contain porcine.

Furthermore, the test was also done for unlabelled samples from traditional market (sample C1-C4). Based on Fig. 5, the positive control showed a rise above threshold line at 72.94, with Ct value 16.19 and RFU 2037, respectively. Sample with code C1, C2, and C3 did not produce amplicon, while sample C4 did, with Ct value 25.35 and RFU value 750. This result was also confirmed by MCA (Fig. 6) which sample C4 showed a peak at T_m 78.5 °C and electrophoresis analysis (Fig. 7). This result implied that from the eight tested samples, one sample was positive containing porcine meat. Porcine DNA was not detected in all of halal labeled samples.

CONCLUSION

Based on this study, it can be concluded that the RT-PCR detection method using ND5 primers (forward primer CATTGCCTCACTCACATTAACC 5'-3' and reverse primer AAGAGAGAGTTCTACGGTCTGTAG 5'-3') proved to be a powerful and simple technique, specific and sensitive for porcine meat detection. The validated method allowed the detection of porcine meat with the limit of detection for no less than 1%. The applicability of the method was demonstrated by the analysis of commercially available meatball products. From eight samples tested, one was positively contained porcine meat. The method was strongly potential to be used in regulatory and enforcement

bodied and quality control for food labelling or Halal verification and certification.

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