The Effect of Boiling Temperature in Beef and Pork Meatball Making Process on DNA Detection using Real Time Polymerase Chain Reaction (RT-PCR) Method

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Abstract. Boiling meat in meatball making process can cause DNA degradation. This study aims at determining the effect of boiling temperature on beef and pork DNA detection in meatball samples using Real Time Polymerase Chain Reaction (RT-PCR). DNA of beef, pork, beef meatballs, pork meatballs and beef-pork meatballs boiled at 8° C, 90° C, and 100° C were isolated using the Wizard Genomic DNA Purification Kit and amplified using the RT-PCR. The result has shown DNA of beef meatball, pork meatball, and beef-pork meatball samples heated to 80º C, 90° C, 100° C can be amplified. Here, the Cycle threshold (Ct) value of the beef meatball, pork meatball, and beef-pork meatball samples heated to 80 °C, 90 °C, 100 °C is significantly different from that of unheated beef and pork ($p < 0.05$). However, there is no significant difference in the Ct value of the beef meatball, pork meatball, and beef-pork meatball samples heated to 80 ° C, 90 ° C, 100 ° C ($p \geq 0.05$). It can be concluded that the beef and pork DNA in meatballs boiled at 80 ºC to 100 ºC is detectable by the RT-PCR.

Keywords: Beef, Heating, Pork, RT-PCR, Temperature

1 Introduction

Indonesia is a country with the largest Muslim population. For Muslims, the concept of *halal* and *haram* is a fundamental law in food consumption, in particular. Pork, for example, clearly *haram* (unlawful/forbidden) food. However, mixing pork with meat-based food is common in Indonesia to gain greater profit. This certainly raises concerns for Muslim community.

In 2019, the Gunung Kidul police officers arrested two suppliers for selling mixed beef and pork [1]. The same case happened in 2017 in Jember where a supplier of mixed beef and pork was taken into custody by the local police [2]. In the same year, the beef contamination with pork was found in branded beef sausages sold at Kranggan Market, Bringharjo Market and Pathuk Market in Yogyakarta[3]. To monitor halal products sold in the markets, an accurate analysis, especially regarding the pork and beef contents in various products is necessary.

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Some of the methods used to analyze the pork content in food samples are UV-Vis Spectroscopy [4], Spectrophotometry Fourier-Transform Infrared (FTIR) [5], Enzyme-Linked Immunosorbent Assay (ELISA) [6], Gold Nanoparticle [7], and Real Time Polymerase Chain Reaction (RT-PCR) [8]. Compared to other methods, the RT-PCR is often applied to analyze the pork content in food products due to its accuracy and specification in DNA detection [9]. It can even carry out sensitive DNA detection as low a concentrations as 1pg/ml [10]. Such technique is suitable for heated products as small fragments of DNA can be identified and detected [11].

An interesting sample to determine for its halal content is meatballs. Meatballs are very popular among the majority of Indonesians. In the meatball making, the boiling process must be carried out to ripen the meat. Traditionally, meatballs are made by boiling the meat and spices at a temperature ranging from 80ºC to 100ºC. In fact, heating the meat can cause DNA degradation. For this reason, this study attempts to analyze the effect of heating at 80ºC to 100ºC on beef and pork DNA detection in meatball products using the RT-PCR method.

2 Method

2.1 Materials

The materials include beef, pork, wheat flour, salt, garlic, pepper, PureLink® Genomic DNA Mini Kit, SensiFAST SYBR No-Rox (Bioline), isopropanolol absolute (Merk, Germany), NaOH (Merk, Germany), ethanol, aquabidest, and a pair of primer beef and pork.

Table 1. Primary base [12]

Primer	Base
Beef Forward	5’- CCCGATTCTTCGCTTTCCAT -3’
Beef Reverse	5’- CTACGTCTGAGGAAATTCCTGTTG -3’
Pork Forward	5’ - CTTGCAAATCCTAACAGGCCTG -3’
Pork Reverse	5’ - CGTTTGCATGTAGATAGCGAATAAC -3’

2.2 Instrument

The instruments applied in this study include the Real Time PCR (q-Tower), multiwell plate 96 (Roche®), 1.5 volume microcentrifuge tube (Biogenix), micropipette (Biorad), centrifugator (5417R-Eppendrof), vortex (Horiba, Japan), digital waterbath (SB-100 Eyela), UV DNA spectrophotometer (DeNovix®), and analytical scales (Horiba, Japan).

2.3 Procedure

Meatballs Making

Beef, pork, the combination of beef and pork (9 grams each) were mixed with 1 gram of flour. Pepper and salt were then added. All ingredients were stirred until they were all mixed up and round like balls. The samples were boiled at 80 °C, 90 °C, and 100 °C for 1 hour.

DNA Isolate

Animal tissue preparation and cell lysis

20 mg of ground meatballs was put into a 1.5 ml microcentrifugation tube and added with 600µL of nucleic lysis solution. The mixture was homogenized with a vortex for 15 seconds and incubated for 30 minutes at 65°C.

Cell lysis and Protein Precipitation.

3µL of RNAse solution was added to the incubated mixture. In turn, the mixture was incubated again at 37°C for 30 minutes. In the next step, 200 µL of protein precipitation solution was added and vortexed. The mixture was let stand for 5 minutes at 5°C before it was centrifuged for 5 minutes at 16000 rpm speed.

***DNA Precipitation and Rehydration*.**

The precipitate and supernatant formed from the centrifugation were separated. 200µL of supernatant was taken and 600µL of isopropanol was then added. The resulting mixture was homogenized and centrifuged for 1 minute at 16,000 rpm speed. The precipitate and the supernatant formed from the centrifugation were separated. The precipitate was added with 600µL of 70% ethanol, homogenized and centrifuged for 1 minute at 16,000 rpm speed. The ethanol-containing precipitate was evaporated using a hairdryer and in turn 50µL of DNA rehydration solution was added.

DNA Isolate Analysis with UV Spectrophotometry

The isolated DNA was analyzed using UV DNA Spectrophotometry. The DNA rehydration solution was used as a blank. About 1µl was placed on the sample port and was then analyzed. The sample port was cleaned and 1µl of the DNA sample was placed on the sample port again. The DNA sample was analyzed at 260 nm and 280 nm wavelengths.

DNA amplification using Real-Time PCR

The amplification was carried out with the composition of solutions shown in Table 2.

Table 2. RT-PCR Mix Composition

Reagent	Volume
Sensi FAST SYBR No-ROX	5 µl
10 µM *forward* primer	0.3 µl
10 µM *reverse* primer	0.3 µl
Template	1.4 µl
Aquadest	3 µl

Data Analysis

The data obtained from the RT-PCR is the Ct value of the amplification process. A statistical analysis was carried out to examine the Ct value. The analysis here includes the parametric test if the normality and homogeneity of variance is met or the nonparametric test if the normality and homogeneity of variance is not met. In this study, the parametric test was conducted with One Way Analysis of Variance (ANOVA), while the nonparametric test was carried out using the Kruskall Wallis Test.

3 Result

The result of the purity test is illustrated in Table 3. The highest concentration was found in control beef DNA (164,648 ng/µl) and the lowest concentration was seen in beef DNA heated to 100 °C (6.8 ng/µl).

Table 3. The results of measurements of the concentration and purity of DNA isolates

No.	Sample	Concentration (ng/µl)	Purity (λ 260 nm/ 280nm)
1.	Beef	164.684	1.430
2.	Pork	15.050	1.320
3.	Beef 80°C	9.43	2.128
4.	Beef 90°C	8.0	1.593
5.	Beef 100°C	6.8	2.4
6.	Pork 80°C	54.33	1.734
7.	Pork 90°C	7.0	1.750
8.	Pork 100°C	17.45	1.700
9.	Beef meatball 80°C	14.00	1.781
10.	Beef meatball 90°C	25.81	1.896
11.	Beef meatball 100°C	17.70	1.654
12.	Pork meatball 80°C	34.75	1.760
13.	Pork meatball 90°C	46.40	1.758
14.	Pork meatball 100°C	13.65	1.784
15.	Mixed meatball 80°C	23.99	1.847
16.	Mixed meatball 90°C	30.94	1.827
17.	Mixed meatball 100°C	34.80	1.758

The Ct value from amplification of beef, pork, beef meatballs, pork meatballs and mixed meatballs heated at 80ºC, 90ºC and 100ºC can be seen in Table 4.

Table 4. Value of Ct amplification process and Standard Deviation (SD)

No.	Sampel	Ct	SD ±
1.	Beef	21.56	0.09
2.	Pork	18.08	0.01
3.	Beef 80°C	31.65	0.50
4.	Beef 90°C	31.59	0.40
5.	Beef 100°C	31.42	0.02

6.	Pork 80°C	26.57	0.50
7.	Pork 90°C	21.35	0.14
8.	Pork 100°C	20.66	0.04
9.	Beef meatball 80°C	30.61	0.94
10.	Beef meatball 90°C	31.37	0.14
11.	Beef meatball 100°C	30.16	0.04
12.	Pork meatball 80°C	24.97	1.78
13.	Pork meatball 90°C	19.16	0.46
14.	Pork meatball 100°C	22.30	0.10
15.	Mixed meatball with bovine primer 80°C	33.23	1.20
16.	Mixed meatball with bovine primer 90°C	31.56	0.31
17.	Mixed meatball with bovine primer 100°C	32.15	0.21
18.	Mixed meatball with porcine primer 80°C	21.77	0.11
19.	Mixed meatball with porcine primer 90°C	22.96	0.67
20.	Mixed meatball with porcine primer 100°C	25.95	2.83

4 Discussion

DNA Isolate

The DNA isolate was carried out on beef and pork (as a control), beef meatballs, pork meatballs, and beef-pork meatballs using Wizard Genomic DNA extraction kit. The isolation process consists of cell and nucleus breakdown process, RNA degradation, protein precipitation, DNA precipitation and purification, and DNA rehydration. The cell and nucleus breakdown process is a process of destroying the integrity of the cell wall barrier. In this process, the nucleic lysis solution was used. In the DNA degradation step, RNAse as an enzyme that degrades RNA was given to remove it in solution [13]. The Next step was adding protein precipitation solution (PPS) to precipitate the protein so that the DNA strands were no longer coiled [14]. The DNA precipitation was carried out by adding isopropanol to the resulting supernatant. The DNA would appear as fine white threads [15]. Then, the precipitate was washed using 70% ethanol in order to make the DNA precipitate free from isopropanol and any remaining salt from the previous process [16]. The final stage was the resuspension of the DNA isolate by providing a DNA rehydration solution to the dried precipitate in order to turn the precipitate formed into a solution easier to examine.

DNA Purity Test

The DNA purity test was conducted to ensure the completion of the amplification process. The UV spectrophotometry at 260 nm and 280 nm wavelengths was used in performing the DNA isolate purity test. Nucleotides have a maximum absorption at 260 nm wavelength, whereas proteins have a maximum absorption at 280 nm wavelength [17]. A good purity value is obtained if the absorption ratio at 260 and 280 wavelengths is in 1.8 - 2.0 range. DNA that has a purity above 1.0 is even acceptable and analyzed using the real-time PCR continues [18]. Based on the purity value, all samples could be continued in the PCR process.

Primary Specificity

Beef and pork primers with forward and reverse sequencing primers were used in Tanabe's study in 2007 [12]. There was 50% of GC in in the forward bovine primer, and 45% of GC in the reverse primer respectively. In the meantime, there was 50% of GC in the forward porcine primer, and the reverse primer contained 40% of GC base. DNA that contains more GC bases will have more stable than the DNA with AT bases [19]. In 2017, Zilhadia conducted a specificity test on pork and beef primers using the NCBI BLAST [20]. However, in this study, the specificity test was carried out with agarose gel electrophoresis. The electrophoresis result showed the two pairs of primers used were specific for beef and pork DNA (the electrophoresis images are not shown).

Determination of Optimal Amplification Method

The temperature optimization was conducted for denaturation, annealing, and extension processes. The modification of annealing temperature affects the primer attachment process to the DNA template. If the annealing temperature is too high, the primer is unable to attach properly to the template. However, if the annealing temperature is too low, the primer will stick to an unspecified attachment site which will then amplify unexpected locus fragments [21]. The annealing temperature is determined by calculating the Tm, which is usually calculated based on (Tm-5)ºC to (Tm + 5)ºC [22]. The Tm generated from the primary protocol for forward and reverse beef primers was 55.4°C, forward pork primers about 56.4°C and reverse primers about 54.3°C respectively. At the annealing stage, the temperature used is equal to 60ºC. In the meantime, the denaturation and extension temperatures refer to the SensiFAST SYBR No-ROX Kit protocol, 95ºC and 72ºC respectively. The determination of the optimal concentration of DNA isolate is needed for some considerations. If it is too high, it can increase the chance of mispriming. However, if it is too low, the primer may not reach the target. [23]. The concentration range in this study was made equal, at a concentration value of 5-10 ng/µl.

The DNA Amplification of Control Beef and Pork, Beef Meatballs, Pork Meatballs and Beef-Pork Meatballs Heated to 80ºC, 90ºC and 100ºC

The Ct value of the control beef DNA amplification process using beef primer is 21.76, whereas the Ct value of pork DNA amplification process using pork primer is 18.08. The Ct value has indicated the control beef and pork DNA amplification process went well. As indicated by the Ct value, the pork DNA amplification was slightly lower than the beef DNA amplification.

The RT-PCR test was carried out on 3 samples of beef and pork heated to 80°C, 90°C, and 100°C respectively. The Ct value in beef samples with beef primer at 80°C, 90°C, 100°C was 31.65 ± 0.50, 31.59 ± 0.40 and 31.42 ± 0.02, respectively. The Ct value in pork samples with pork primer at 80 °C, 90 °C, 100 °C was 26.57 ± 0.5.38, 21.35 ± 0.14 and 20.66 ± 0.004, respectively. The Ct value of heated beef and pork was higher than that of unheated beef and pork. This shows the heating process has given an influence on the beef and pork DNA amplification process. According to the theory, heating will cause DNA degradation, but the DNA can still be amplified using polymerase enzymes. However, there was no significant difference in the Ct value of beef and pork heated to 80ºC, 90ºC and 100ºC. This means temperature differences ranging from 80ºC to 100ºC did give any significant effect.

The next amplification was performed on 3 samples of beef meatballs and pork meatballs heated to 80°C, 90°C, and 100°C. The Ct value of the beef meatball samples with beef primers at

80°C, 90°C, 100°C was 30.61 ± 0.94, 31.37 ± 0.14, and 30.16 ± 0.04 respectively. Meanwhile, the Ct value of pork meatball samples with pork primer was 80°C, 90°C, 100°C was 24.97 ± 7.78, 19.16 ± 0.46, 22.30 ± 0.10, respectively. The Ct value in the amplification results of beef and pork meatballs at 80 ºC-100 ºC range which was statistically tested indicated no significant difference. The same case happened to the mixed beef and pork meatballs. The Ct value of the mixed meatball samples with bovine primer at 80°C, 90°C, 100°C was 33.23 ± 1.20, 31.56 ± 0.31, and 32.15 ± 0.21 respectively, whereas the Ct value with porcine primer was equal to 21.77 ± 0.11, 22.96 ± 0.67, and 25.95 ± 4.83, respectively.

Sakalar et al, 2012 conducted some test on beef, pork, and chicken by heating the samples in an oven to 30°C, 60°C, 90°C, 120°C, 150°C, 180°C, and 210°C. The test discovered the temperature and heating duration affected the results of Real Time PCR detection; the higher the heating temperature and time, the higher the amplification value of the sample [24]. However, this study has shown different results probably due to the close temperature ranges. In the study conducted by Karni et al in 2013, a DNA degradation test was made using the agarose electrophoresis method in which the pUC19 DNA isolates were heated. The heating was made at 95ºC, 130ºC, 140ºC, 150ºC, 160ºC, 170ºC, 180ºC, and 190ºC respectively. As the result, DNA began to degrade at 130°C and reached complete degradation at 190°C [25]. Boiling in the process of making meatballs at 80°C to 100°C did not affect the Ct value in the amplification process. In this regard, the analysis of differences in beef and pork can still be conducted with good degree of accuracy.

5 Conclusions

Referring to the results and discussion, boiling beef, pork, beef meatballs, pork meatballs and beef-pork meatballs at 80°C, 90°C, and 100°C is significantly different from the unheated beef and pork ($p \leq 0.05$). However, the heating process in beef, pork, beef meatball, pork meatball, and beef-pork meatball samples at different temperature (80°C, 90°C, and 100°C) did not show any significant difference ($p \geq 0.05$). This means the DNA heated at 100ºC could be amplified. For this reason, mixing pork and beef in the process of making meatballs heated to 100ºC was detectable and analyzable.

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