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DNA-based detection of Rat in the meatballs product using a real-time polymerase chain reaction method

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ABSTRACT

The meat-based products are highly susceptible to counterfeiting, primarily due to high consumer demand of meat derivative products, such as meatballs. This demand creates opportunities for food fraud by specific industries, including adulterating meat with non-halal species, such as rats. This research aimed to detect rat meat contamination in meatball samples from the Indonesian local market using Real-time Polymerase Chain Reaction (RT-PCR). The RT-PCR amplification involved an initial denaturation step at 95°C for 3 minutes, followed by denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. The rat-specific probe primer included in the kit produced an increasing curve in the External Positive Control (EPC) with a Ct value of 27.22, and no amplification occurred in the Negative Control (NTC). The analysis of 30 samples from meatball vendors yielded negative results, as there was no increase in the FAM (rat) curve, indicating that none of the meatballs were contaminated with rat DNA.

Keywords: food fraud, meatballs, Rat, Real-time PCR, meat-based products

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INTRODUCTION

Nowadays, public halal awareness in Indonesia is rising as food frauds and adulterations increase. The increasing awareness is also affected by the implementation of the Indonesian Halal Regulation on Law Number 33 of 2014 concerning Halal Product Assurance. However, food adulteration and fraud incidents have continually occurred (Nida et al., 2020). These cases also happen globally and threaten the market (Owolabi & Olayinka, 2021). In the case of meat-based products the high consumer demand for meat-based products has led several industries to commit fraud in terms of mixing meat with non-halal materials. Meatball fraud is the most common food fraud in the Indonesian market. Meatballs are one of the popular dishes in Indonesia (Purnomo & Rahardiyan, 2008). The primary ingredient is beef; however, the beef is mixed or replaced with other animals at relatively lower prices to reduce costs, such as chicken, pig, boar, or rats.

It has been reported that 7.83% of beef samples were mixed with boar meat throughout 2013-2017 in Bogor (Nida et al., 2020). A study of commercial beef meatballs reported that 22 of 36 meatballs samples in Bojonegoro, East Java, contained pork (Siswara et al., 2022). Nine cases of pork contamination in the meatball samples were reported in Yogyakarta Province (Erwanto et al., 2014). Other studies also revealed pork contamination in Indonesian meatballs (Cahyadi et al., 2020; Indriati & Yuniarsih, 2019; Siswara et al., 2021; Waluyo et al., 2023). In addition to pork contamination, dog meat was also found in the adulterated beef meatball formulation (Guntarti & Purbowati, 2019; Rohman, Pebriyanti, et al., 2020; Rohman, Rahayu, et al., 2020). Recently, the cases of rat meat contamination have also increased in meatball products (Cahyadi et al., 2020; IkaWidyasa et al., 2015; Lestari et al., 2022; Suryawan et al., 2020). In addition, other processed meat products, including sausages, are also reported to contain rat meat (Sunaryo et al., 2022).

As halal authenticity is essential, various detection techniques have been elaborared to confirm the presence of non-halal species in meat-based products, like meatballs. The most common and accurate technique was Polymerase Chain Reaction (PCR). To conduct the PCR technique, the DNA contained in the samples was extracted (Sunaryo et al., 2023). There are many studies on pork detection in meatball samples by conventional PCR (Cahyadi et al., 2020; Erwanto et al., 2014; Indriati & Yuniarsih, 2019). In those studies, several gene targets were used to detect the presence of porcine genomics, such as cytochrome-b-genes and 12S rRNA genes (Cahyadi et al., 2020, 2021). A real-time PCR was also used to test such meat contaminations since the method provides faster and more reliable detection of meat-based products (Dalsecco et al., 2018). It has been reported that RT-PCR has high sensitivity and specificity over other methods; thus, it is used as a standard halal authentication analysis and frequently employed to detect traces of pork in foods (Hibaturrahman et al., 2023; Rohman, Rahayu, et al., 2020; Deepak et al., 2007). The pork detections by Real-time PCR were also reported (Mustaqimah et al., 2021; Raharjo et al., 2017; Salamah et al., 2019; Waluyo et al., 2023). Meanwhile, several studies on rat detection were conducted by PCR using several genes target of rats, such as mitochondrial cytochrome-b-genes of *Rattus argentiventer* and the Mt-atp6 genes of *Rattus norvegicus* (IkaWidyasa et al., 2015; Masnaini et al., 2023; Sihotang et al., 2023; Sunaryo et al., 2022; Suryawan et al., 2020). Some protein markers of *Rattus norvegicus* were also used to detect rat contamination in meat-based products (Aini et al., 2022).

In this study, we used a real-time PCR technique to detect rat meat contaminations on the meatball samples from the Indonesian local market in DKI Jakarta Province. Prior to the PCR test, the DNA samples were extracted using *the Progenus EasyFast™ Extraction Kit for Meat Products*, as described in the previous study (Sunaryo et al., 2023). This study is of urgent importance as the research on rat detection in the meatball samples using a real-time PCR is yet limited. The result of this study will significantly contribute to public halal awareness of meatball products that are commercially distributed in the local Indonesian market.

MATERIALS AND METHOD

Materials

The instruments were micropippet (Bio-Rad™), mortar set, centrifuge (Thermoscientific™), heat block (My block, Benchmark), NanoDrop 2000 spectrophotometer (Thermoscientific™), Real-time PCR from CFX96 Deep Well, Bio-Rad™, and analytical balance. The materials were a 30-samples of meatballs from a local Indonesian market around DKI Jakarta Province, a *Progenus EASYFAST™ kit for Rat Detection kit, a Progenus EasyFast™ Extraction Kit for Meat Products, rat meat as a positive control, PCR tubes, and 1.5 mL microtubes.*

Sample collections

This study obtained 30 DNA sample from meatballs in Cengkareng district, DKI Jakarta Province, Indonesia. All samples were labeled, and their DNA was extracted directly. The rat meat was used as a standard. The standard DNA was also extracted and prepared for PCR amplification.

DNA extractions

The genomic DNA of meatballs and rat meat was extracted by kit, as described by Sunaryo et al. 2023. The first step began with solution A. Solution A was added to sample tubes. The solution was homogenized and heated at 95°C for 1 hour. It was then continued by adding solution B. All the solutions in the microtubes were mixed. The supernatant was transferred to the microtube. The final solution was diluted with 10X of nuclease-free water. The extracted DNA was analyzed by a NanoDrop 2000 spectrophotometer.

Measurement of test purity and DNA content

The purity of the DNA sample was analyzed using a spectrophotometer. The measurements were performed in the wavelength of 260 nm, 280 nm, and 260/280 nm (Sunaryo et al., 2023).

Amplification with the real-time PCR

The RT-PCR process was conducted using CFX96 Deep Well by Bio-Rad™, USA, as described in the previous study (Sunaryo et al., 2023). All the PCR tubes were prepared. Each reaction mixture was prepared by adding MIX reagent (18 µL) and either the samples or External Positive Control (EPC) or rat meat as standard or Nuclease-free water as negative control (2 µL), in order to give the total reaction volume as 20 µL. All the tubes were then put in the PCR instrument. The PCR was performed by setting in all the parameters: the cycle of pre-denaturation and denaturation-annealing-extension step. The pre-denaturation was conducted at 95°C - 3 minutes. The denaturation-annealing-extension was carried out at 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing-extension). The FAM value at 494/520 nm represents *Rattus* species. The VIC value at 538/554 nm represents the gene target for vertebrates (internal control).

Analysis of real-time PCR result

The data obtained in this study was based on identifying DNA rats in the meatball samples. The primary data was analyzed descriptively and presented in the cycle threshold (Ct) value based on the fluorescence results on the Real-time PCR instruments.

RESULT AND DISCUSSION

Meat consumption in Indonesia increases yearly and is accompanied by an increasing need for protection against falsely labeled food. Even though halal labels on meat products are mandatory as an implementation of Indonesian halal regulations, there are still many incidents of food fraud and

adulterations. Therefore, public halal awareness demands a vast exploration of the detection of the non-halal material in foods.

The most common method used for detection in foods, especially meat-based products, is Polymerase Chain Reaction (PCR). Apart from the conventional PCR, the Real-time PCR is also widely used for detection. The RT-PCR method for meat-fraud detection is very accurate, sensitive, and efficient in detecting the presence of DNA content in the samples. The method was chosen as the amount of DNA amplified can be directly observed without gel electrophoresis analysis. In this study, 30 samples of meatballs were collected from the meatball sellers around Cengkareng district, DKI Jakarta Province. All samples were collected by accessible population sampling, which requires at least 30 samples as a minimum for sufficient research (Thomson, 2011).

The DNA extraction was the first step prior to the PCR amplification. In this study, we extracted DNA for all meatball products as samples and rat meat as a standard. The extracted DNA was used as a template for amplification. The parameters, which were purity and concentration of DNA, were analyzed using a Nanodrop 2000 spectrophotometer. The ratio in the absorbance of 260nm and 280nm were used to analyze the DNA quality (Glasel, 1995). It is known that the recommended protein standard range was suggested by an A260/280 ratio of 1.70-2.00 (Adriany et al., 2020). However, an A260/280 ratio above 1.0 is acceptable for the analysis to continue using real-time PCR (Priyanka et al., 2017). The results of the DNA quantitative analysis described that the extraction and isolation process involved sufficient steps, yielding in a pure DNA sample. It is known that high DNA purity can affect the validity of the PCR method. Therefore, analyzing the quality and quantity of DNA was an important and crucial step.

In this study, the ratio samples of A260/280 were in the range of 1.28-2.24, as shown in Table 1. The obtained A260 value is the DNA absorbed at the wavelength of 260 nm. Meanwhile, the A280 value at a wavelength of 280 nm indicates the presence of contaminations. Therefore, the high or low value of A260 is very critical for DNA purity. The highest value of A260 does not always mean that the purity of the DNA is high; rather, it can be influenced by the A280 value of contaminations. Table 1 shows that the purity value of the meatballs was within normal limits. However, some were less or more than the ideal value. This result is probably caused by some reagent components, such as phenol, alcohol, and chloroform at extraction time. Other factors that can affect the DNA's quality and purity are the presence of protein, RNA, and other impurities that come originally from the sample.

The concentration of the DNA is one of the critical factors during PCR. If the amount of the DNA is relatively low, the PCR yield will not be optimum. According to the previous study, the recommendation of DNA concentration required for PCR ranged from 10-100 µg/mL, whereas another study stated that the optimum concentration in PCR with 30 cycles was 50 µg/mL (Maryam et al., 2016; Nugroho et al., 2017). In this study, the DNA concentration extracted from the meatball sample was relatively low. It happened because of the added ingredients that were mixed in the samples. Ingredients such as flour, spices, and other ingredients can probably disrupt the extraction process. Certain procedures in meatball production can produce difficulties during DNA extraction. According to the previous study, those procedures might disrupt DNA extraction, such as grinding meat, heating treatment at a very high temperature, and mixing additives in the meatballs (Indriati & Yuniarsih, 2019).

It is reported that the extraction of DNA can be performed from meat that has been heating at 100°C and 120°C for 30 minutes (Matsunaga et al., 1999). The previous study successfully extracted and amplified DNA from meat-based products such as sausages, corned beef, meatballs, and beef jerky; in other words, the DNA was not destructed by heating (Nuraini, 2004). A study stated that PCR amplification was not affected by adding ingredients or cooking processes (Martín et al., 2007). Another study successfully amplified 12S rRNA in genomic samples of several animals heated at high temperatures (Kesmen et al., 2007). However, our data on the extracted DNA in this study showed good purity and concentration. To avoid the unsuccessful in amplifying the DNA of meatball samples in this study, the PCR was performed at a 40-cycles.

The obtained DNA samples were then run by real-time PCR. The analysis data of 30 samples of meatball products were shown as *Cycle Threshold* (Ct) values. The number of cycles needed to replicate the DNA content to be detected crossing a threshold is considered the Ct value. The higher Ct value indicates a smaller amount of DNA/RNA in the samples, suggesting that more cycles are needed for detection. Meanwhile, the low Ct value represents a larger amount of DNA/RNA in the sample, indicating that fewer cycles are needed for detection. However, if the gene target on the samples is absent, the value was shown as Not Applicable (N/A).

The *External Positive Control (EPC)* from the kit contains the gene target of rats and vertebrates, whereas the control negative contains *Nuclease Free Water (NFW)*. The EPC and negative control in this study showed valid results following the kit recommendations, in which the Ct value of both VIC and FAM were less than 30 for positive controls, and the Ct value of both VIC and FAM were more than 38 for negative controls, as shown in Table 2 and Figure 1.

Table 1. The parameters of DNA in standard and meatball samples by Nanodrop 2000 Spectrophotometer

Samples	Purity A260/280 (nm)	Concentration (ng/mL)
ST	1.87	3045.9
PB1	1.57	2789.6
PB2	1.91	2130.6
PB3	1.64	3027.6
PB4	1.82	1831.1
PB5	1.88	3027.5
PB6	1.95	1827.2
PB7	2.24	2684.1
PB8	1.79	2817.9
PB9	1.81	2141.8
PB10	1.28	1005.4
PB11	1.99	1627.4
PB12	1.78	1268.3
PB13	2.21	1458.4
PB14	1.76	2916.4
PB15	1.88	1629.2
PB16	1.79	3364.8
PB17	1.86	1418.2
PB18	1.77	1922.6
PB19	2.15	1404.7
PB20	2.04	2132.4
PB21	1.80	1862.4
PB22	1.53	1507.3
PB23	1.96	2086.8
PB24	1.78	2284.1
PB25	1.82	1949.3
PB26	1.80	2466.4
PB27	1.81	1418.2
PB28	1.72	2389.7
PB29	1.60	1730.3
PB30	1.79	3067.4

Notes: ST (standard contains rat meat); PB (meatballs samples)

The amplification curves of EPC and NFW was shown by Figure 1, which indicates a sigmoid increase in the curve of VIC (shown in yellow) for vertebrates and FAM (shown in blue) for rats. Meanwhile, there is no increase in the sigmoidal curve for the control negative in both VIC and FAM. This result alligns the literature that the positive control is characterized by a fluorescent signal crossing the baseline threshold. Meanwhile, the curve of negative control does not show the increase in the sigmoidal curve and does not pass the baseline threshold. The validity of this study based on the positive and negative control results is in accordance with similar studies (IkaWidyasa et al., 2015; Rohman, Rahayu, et al., 2020). Therefore, it can be concluded that the experiment in this study was in good condition, and the work process was good.

Table 2. The Ct values (VIC and FAM) for positive and negative control, rat meat, and meatball samples

Samples	Ct value	
	VIC	FAM
EPC	28.84	27.22
NFW	N/A	N/A
Standard	14.97	14.27
PB1	21.98	N/A
PB2	21.95	N/A
PB3	21.97	N/A
PB4	16.27	N/A
PB5	16.26	N/A
PB6	16.25	N/A
PB7	20.50	N/A
PB8	20.51	N/A
PB9	20.48	N/A
PB10	18.75	N/A
PB11	18.73	N/A
PB12	18.76	N/A
PB13	17.19	N/A
PB14	17.21	N/A
PB15	17.20	N/A
PB16	18.95	N/A
PB17	18.91	N/A
PB18	18.96	N/A
PB19	18.32	N/A
PB20	18.35	N/A
PB21	18.33	N/A
PB22	18.95	N/A
PB23	18.96	N/A
PB24	18.95	N/A
PB25	18.64	N/A
PB26	18.62	N/A
PB27	18.61	N/A
PB28	17.86	N/A
PB29	17.59	N/A
PB30	17.93	N/A

Notes: EPC (External Positive Control); NFW (Nuclease Free Water); PB (meatballs samples)

In addition, Figure 2 showed that the Ct values of both VIC and FAM from the pure rat meat as standard were 14.97 and 14.27, as shown in Table 2 and Figure 2, suggesting that the DNA rat in the standard is abundant. This result showed that the analysis sample of rat meat using Real-time PCR was well detected.

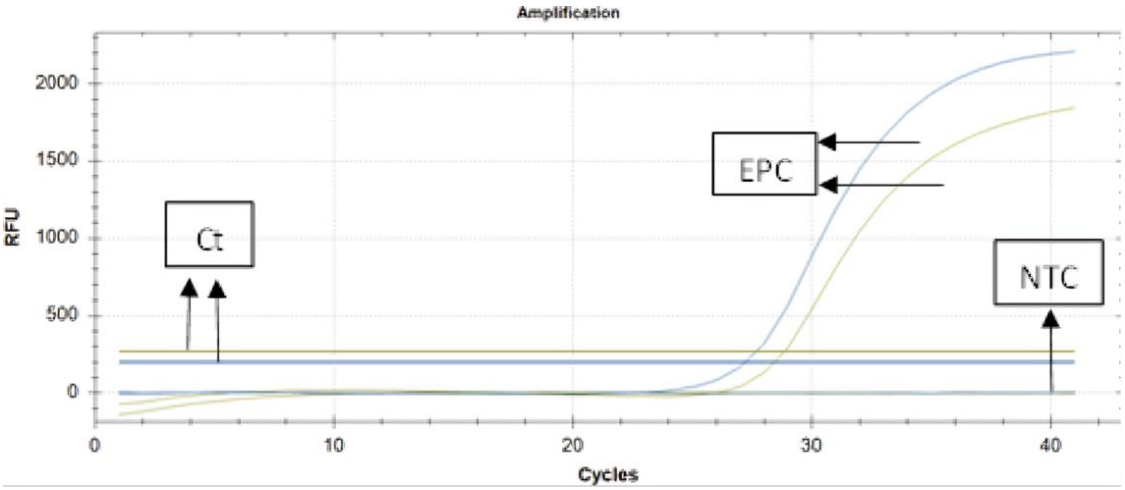

Figure 1. The amplification curves of external positive control (EPC) and nuclease free water (NFW) as negative control (NTC) from progenus easyfast™ kit. the VIC value is shown in yellow. The FAM value is shown in blue

However, according to the amplification results from the samples shown in Table 2 and Figure 3, all meatball samples from Cengkareng district, DKI Jakarta Province, did not contain the DNA of rat meat. The Ct value of all samples for VIC was less than 30, and FAM was more than 38 or as Not Applicable (N/A), suggesting that all samples contain the gene target of vertebrate, but the DNA of rat meats was not detected in all of the meatball samples.

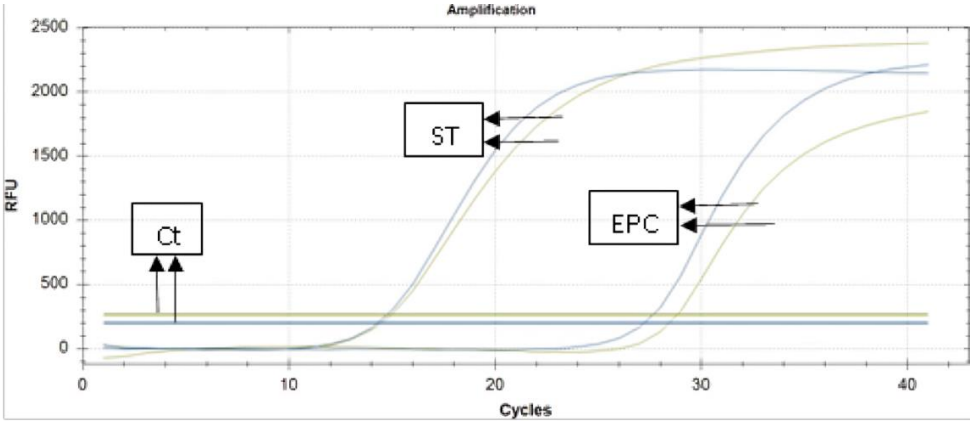

Figure 2. The amplification curves of rat meat as a standard (ST) and External Positive Control (EPC) from progenus easyfast™ kit. the VIC value is shown in yellow. The FAM value is shown in blue

Figure 3 shows the representative result of the amplification curve from the *Progenus EasyFast*TM kit control and meatball sample (PB2) with a purity value of A260/280 of 1.91. The PB2 of the

meatball sample showed a sigmoidal increase in the VIC curve (yellow) to detect vertebrates, but there was no increase in FAM (blue). This result exhibited that the meatball samples analyzed using real-time PCR were negative for rats, but there were vertebrates with a Ct value of 21.95 in the meatball samples. The VIC value of the samples indicated that the meatball samples contained vertebrates that possibly originated from beef meat. A similar study showed no positive contaminant in the samples (Indriati & Yuniarsih, 2019).

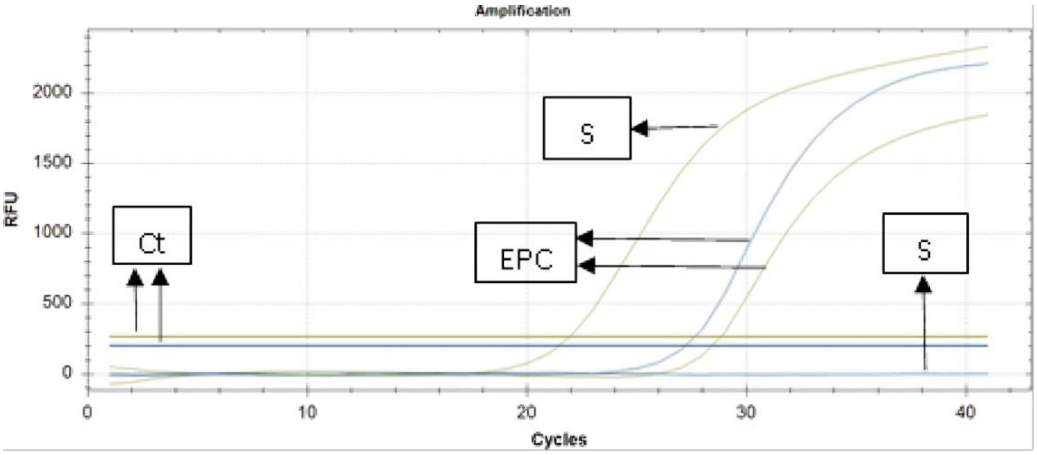

Figure 3. The amplification curves of external positive control (EPC) and PB2 samples (S) from progenus easyfast™ kit. The VIC value is shown in yellow. The FAM value is shown in blue. The VIC and FAM values of samples were shown in S yellow and blue

CONCLUSION

Based on the research conducted on the detection of rat DNA contamination in the 30 meatball samples from the Cengkareng district using the Real-time PCR method, no positive results were obtained. Therefore, we concluded that the meatball samples circulating in the area are relatively safe because they do not contain rat DNA.

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