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Research article

Development of Rat Meat Detection Using *Mt-atp6 Rattus norvegicus* Gene Genetic Marker

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detection;

Mt-atp6;

real-time PCR

Abstract

An empirical test using the real-time PCR method to detect food contamination with rat meat (*Rattus norvegicus*) was conducted. The aims of this study was to provide information on new genetic markers that can be used to detect rat DNA. Detection of rat DNA in meatballs using the genetic marker of the *Mt-atp6* (mitochondrial encoded ATP synthase membrane subunit 6) *Rattus norvegicus* gene was carried out using meatballs containing rat meat. Primers and probes were designed using Primer3Plus and then analyzed for in silico specificity using Primer-BLAST and Nucleotide-BLAST. Forward primer (5'-ACACCA AAAGGACGAACCTG-3') , reverse primer (5'- AGAATTACGGCTCCT GCTCA-3'), and probe (5'-[VIC]-TTCTAGGGCTTCTTCCCCAT-[QSY]-3') with a target size of 161 bp, were successfully designed. The results of empirical validation with laboratory experiments showed that the primer and probe pair can detect *Rattus norvegicus* specifically.

1. Introduction

Meatballs are Indonesian culinary commodities made from meat or fish. Some meatballs are processed meats made using beef, chicken, pork, or fish meat, but the most popular type of meatball on the market is the beef meatball [1]. The price of beef in Indonesia is quite high, prompting some sellers to fake beef meatballs with cheaper meat to maximize profits [2]. Substitution of raw materials in processed food products often occurs when manufacturers try to reduce the use and the cost of their scarce or expensive raw materials, which may be expensive due to limited supply. One of the substitute raw materials that can also be used in meatball product is rat meat (*Rattus norvegicus*). The halal aspect of food is very important in Indonesia where the majority of the population is Muslim, thus the main issue of mixing beef meatballs with rat meat must be considered [3]. Regarding the halal aspect, the consumption of wild animals such as rats

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should be avoided as many viruses are found not only in their saliva, urine, and blood but also in their feces [4]. Rodents including rats are a source of several pathogens capable of affecting human health [5]. Rats can cause diseases such as leptospirosis, salmonellosis, Lassa fever, and lymphocytic choriomeningitis in humans [6, 7].

Various testing techniques to detect the use of certain types of meat as raw materials continue to be developed. An example is the use of chemometric techniques [8] and PCR [9-12]. Several studies were conducted to detect rat DNA contamination in meatballs using genetic markers ND1 (NADH dehydrogenase 1) [13], Cytochrome B (Cyt b) [14, 15], and *Mt-atp6* [10, 11]. Rat meat detection in previous studies relied on the use of PCR endpoints and SYBR Green. In this study, a pair of primers and probe will be designed, and then a performance test of the primers and probe pairs will be carried out. The use of the TaqMan probe results in a more sensitive and high specificity method. This study used the genetic marker of the *Mt-atp6 Rattus norvegicus* gene. This gene is a mitochondrially encoded ATP synthase membrane subunit 6 gene. The mitochondrial gene is reliable for fingerprint analysis as it offers sufficient conserved area within the species due to maternal inheritance and because it is available in thousands of copies per cell [10]. The *Mt-atp6* gene functions to provide information to encode the formation of proteins important for normal mitochondrial function [16].

Primer design is an important part of molecular-based research [17]. Specific primer design determines the success of species-specific detection using PCR [18]. The use of the TaqMan probe in the real-time PCR method can increase the specificity and speed of testing. The real-time PCR method allows the observation of test results to be carried out while the DNA multiplication process is in progress [19]. This method is based on the specific hybridization of a probe designed for a particular species with DNA in the sample to be analyzed. Hybridization will only occur in DNA that is complementary to the specific probe [20].

Based on this background, this study was conducted to provide a reference for new genetic markers in the DNA detection test of rat species. In general, there are 2 stages to obtain specific genetic markers. The first stage is to design and analyze the primer and probe sequences in silico, followed by conducting experiments in the laboratory [21]. Results of this study will be used in routine testing to detect rat contamination in foods by first conducting validation or verification of this method to meet the quality assurance of good test results

2. Materials and Methods

2.1 Samples preparation

This study used two types of samples, namely positive and negative samples. Negative samples are also known as negative controls. Positive samples are meatballs made from rat meat. The TaqMan probe and SYBR green used the same positive samples in the real-time PCR assay. A negative control is a sample that does not contain DNA from the species being detected. The negative control for the real-time PCR assay using the TaqMan probe was a mixture of chicken, squid, tuna, guinea pig, and mice (*Mus musculus*). Meanwhile, mice (*Mus musculus*) meat was the negative control for the real-time PCR assay using SYBR green.

Chicken, squid, and tuna were obtained from the market, while rats, guinea pigs, and mice from the National Quality Control Laboratory of Drug and Food, Indonesian FDA. All animals used in this experiment were dead animals. There was no treatment of live animals. There was no violation of the code of ethics.

Negative control meats were boiled for 5 min, then mashed using a blender and stored at -20^{o}C while rat meatballs were made by mincing rat meat and then adding corn starch to form

small balls. Furthermore, the dough that had been shaped into small balls was boiled in boiling water until the meatballs floated in the water. The rat meatballs were then stored at -20ºC. Samples and negative control were stored in separate containers to prevent cross-contamination.

2.2 Primer and probe design

The *Mt-atp6 Rattus norvegicus* gene sequence (NC_001665. 2) was retrieved from the National Center of Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov. Primers and probes were designed using the Primer3Plus software which is available at https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi [22]. The specificity of selected primer pairs was analyzed in silico using Primer-BLAST and Nucleotide-BLAST via the NCBI site [23].

2.3 DNA isolation and amplification

DNA isolation was carried out using the DNeasy *mericon* Food Kit. DNA extraction was carried out on the meatball samples by first homogenizing meatball samples and weighing 2 g for each of ten replications. DNA extraction was performed according to the manufacturer's instructions. The concentration and purity of total extracted DNA samples were determined by measuring absorbance at 260 nm and 280 nm using a spectrophotometer (Thermo Scientific™ NanoDrop 2000) [24].

Target DNA was amplified in a 25 µL reaction consisting of 12.5 µL TaqMan® Fast Universal PCR Master Mix (2x), 1 µL forward primer (7.5 µM), 1 µL reverse primer (7.5 µM), 1 µL Probe (5 µM), 4.5 µL sterile nuclease-free water, and 5 µL DNA template 0.0003 ng/µL. DNA amplification was performed using the QuantStudio 3 Real-Time PCR System (Applied Biosystems) with PCR conditions 20 s of initial denaturation at 95ºC, followed by 45 cycles of denaturation (1 s at 95ºC) and annealing (20 s at 60ºC).

To find out whether the primer worked well without the use of a probe, amplification was performed using SYBR Green. The total volume in one PCR reaction was 25 µL which contained 12.5 µL of PowerUp SYBR Green Master Mix (2x), 1 µL of 3.75 µM forward primer, 1 µL of 3.75 µM reverse primer, 5.5 µL of sterile nuclease-free water, and 5 µL of DNA template addition. The concentration of the added template DNA was 20 ng/µL. The real-time PCR instrument was run using fast mode, with PCR conditions at 50ºC for 2 min UDG activation, DNA polymerase activation at 95ºC for 2 min, 45 PCR cycles with denaturation steps of 95ºC for 1 s, and annealing at 60ºC for 30 s. The melt curve stage was set at 95ºC for 1 s followed by 60ºC for 20 s and 95ºC for 1 s.

Amplification was carried out on positive samples (rat meatballs) and negative control samples. To ensure that there was no contamination in the master mix reagent, amplification of the no template control (NTC) in the form of sterile nuclease-free water was also carried out as a substitute for positive and negative samples. The amplicon from the positive sample containing rat meat was confirmed by sending the amplicon to the 1st BASE for sequencing.

3. Results and Discussion

3.1 In silico analysis specificity of primer and probe

The primer and probe pairs selected for use are shown in Table 1. The primer and probe designs have met the criteria for good primers and probes. Good primer criteria include having a melting

temperature (Tm) of 57-63°C, length of 18-27 bp, the composition of GC in sequences of 45-55%, and maximum 3' self-complementarity of 3.0 [25].

Table 1. Selected primers and probes for the *Mt-atp6 Rattus norvegicus* gene

Sequence (5'→3')	Length	TM (ºC)	GC (%)	Self 3' complementarity
RnATP6-161 Forward: ACACCAAAAGGACGAACCTG	20	60	50	1.0
RnATP6-161 Reverse: AGAATTACGGCTCCTGCTCA	20	60	50	2.0
RnATP6-161 Probe: [VIC]-TTCTAGGGCTTCTTCCCCAT-[QSY]	20	60	50	2.0
PCR size 161 bp				

The relative position of the probe primer pair to the *Mt-atp6 Rattus norvegicus* gene is shown in Figure 1. The primer specificity performed by aligning the primer pair sequences through the Primer- BLAST feature on the NCBI site showed that the primer pair used was able to attach to 203 sequences contained in the NCBI database on October 24, 2021 (Table 2). In addition to *Rattus norvegicus*, the primer pair had the opportunity to recognize 26 other species. All of these species belong to the order Rodentia. Detection of rat species DNA in this study used a probe. The use of probes in the real-time PCR method can increase the specificity of the assay [19]. The working principle of the probe is a specific hybridization designed for a particular species in the sample to be analyzed. Such hybridization will only occur in sequences that are complementary to the specific probe [20].

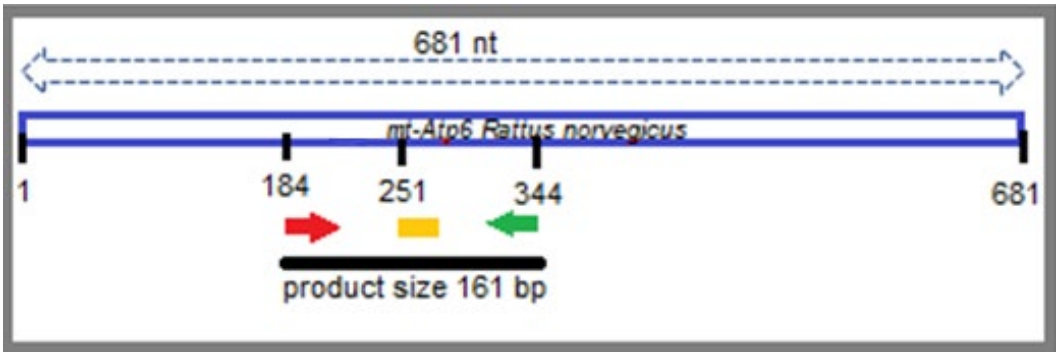

Figure 1. Relative position of the probe primer to the *Mt-atp6 Rattus norvegicus* gene. The red arrows indicate the primary forward position; the yellow box indicates the probe position; the green arrow indicates the reverse primary position.

The results of nucleotide alignment using the Nucleotide -BLAST feature on the NCBI site are shown in Table 3. From Table 3, it can be seen that the nucleotide sequences of PCR products only have similarities with *Rattus norvegicus* with Query Cover and % Identity values of 100% and E-Value of 10^{-76}. Alignment using Nucleotide-BLAST allows searching for sequence similarity to several databases contained in the NCBI GenBank. Query Cover and percent identity

must be 100%. Expectation Value (E-value), which is an indicator of the probability of finding a match by chance, must be 0.01 [26]. The results of the in silico specificity analysis showed that the primer pair and probe had good specificity for detecting rat.

Table 2. Results of the *Mt-atp6 Rattus norvegicus* primer pair sequences alignment

Species	Order	Family	Total
Rattus norvegicus	Rodentia	Muridae	126
Rattus tunneyi	Rodentia	Muridae	1
Rattus lutreolus	Rodentia	Muridae	1
Rattus baluensis	Rodentia	Muridae	20
Rattus rattus	Rodentia	Muridae	4
Rattus tiomanicus	Rodentia	Muridae	6
Rattus tanezumi	Rodentia	Muridae	3
Rattus andamanensis	Rodentia	Muridae	1
Rattus villosissimus	Rodentia	Muridae	1
Rattus exulans	Rodentia	Muridae	14
Zyzomys argurus	Rodentia	Muridae	1
Bandicota bengalensis	Rodentia	Muridae	1
Lophuromys menageshae	Rodentia	Muridae	1
Lophuromys chrysopus	Rodentia	Muridae	1
Maxomys whiteheadi	Rodentia	Muridae	2
Maxomys ochraceiventer	Rodentia	Muridae	1
Grammomys dolichurus	Rodentia	Muridae	1
Sundamys infraluteus	Rodentia	Muridae	3
Leopoldamys edwardsi	Rodentia	Muridae	1
Leopoldamys sabanus	Rodentia	Muridae	2
Niviventer cremoriventer	Rodentia	Muridae	2
Niviventer fulvescens	Rodentia	Muridae	2
Onychomys leucogaster	Rodentia	Cricetidae	1
Rhabdomys dilectus	Rodentia	Muridae	1
Melomys burtoni	Rodentia	Muridae	1
Stenocephalemys zimai	Rodentia	Muridae	4
Malacomys edwardsi	Rodentia	Muridae	1
Total			203

Table 3. Results of PCR product sequence alignment

Amplicon sequence (5'→3')	Number of Target Species Recognized		
	Rattus norvegicus	**Other species**	**Total**
ACACCAAAAGGACGAACCTGA GCCCTAATAATTGTATCCCTAA TTATATTTATTGGCTCAACCAA CCTTCTAGGGCTTCTTCCCCATA CATTTACCCCTACCACTCAGCT ATCTATAAACCTAAGCATAGCC ATCCCCCTATGAGCAGGAGCCG TAATTCT	100 *Query Cover*=100% % *Ident.* = 100% *E-Value* = 10^{-76}	0	100

3.2 Isolated DNA analysis

Total DNA isolated was analyzed for concentration and purity using a microvolume spectrophotometer (Table 4). From Table 4, it can be seen that the concentration values of the extracted samples were in the range of 178.0 ng/µL - 330.2 ng/µL. As for the purity value, which is the wavelength ratio value of A260/A280, it was in the range of 1.90 - 2.00.

Table 4. Concentration and purity of total DNA isolated

Sample	Concentration (ng/ µL)	Purity (A260/A280)
Rat meatball 1	204.3	1.99
Rat meatball 2	206.7	1.98
Rat meatball 3	187.6	1.99
Rat meatball 4	201.4	2.00
Rat meatball 5	202.5	1.98
Rat meatball 6	329.7	1.98
Rat meatball 7	322.4	1.98
Rat meatball 8	330.2	1.98
Rat meatball 9	295.8	1.98
Rat meatball 10	316.6	1.98
Negative control	178.0	1.90

From the data shown in Table 4, it can be seen that all the DNA isolated was of good quality. Good DNA has a purity value in the range of 1.7-2.0 [27]. Thus, the isolated DNA was suitable for analysis using real-time PCR.

3.3 Real-time PCR analysis

The amplification curve using real-time PCR can be seen in Figure 2, and the amplified Ct data is presented in Table 5. From Figure 2, it can be seen that the sample used in the study was amplified in the CT value range of 29.127-29.485. To provide additional data to determine the primer specificity without using a probe, an experimental specificity analysis was also performed on *Mus musculus* using SYBR Green. We can see the amplification curve using SYBR Green and the resulting melt curve in Figure 3 and Figure 4.

Analysis using the real-time PCR method can be carried out using the TaqMan probe or using the SYBR Green [28]. The use of probe provides more specific test results compared to using SYBR Green because the probe will only attach to complementary target sequences. The 5'→3' exonuclease activity of the Taq polymerase enzyme causes the probe to degrade so that the reporter attached to the probe will fluoresce to indicate that amplification has occurred. However, if no target sequence is found that is complementary to the specific probe used, the reporter will not fluoresce [19, 29].

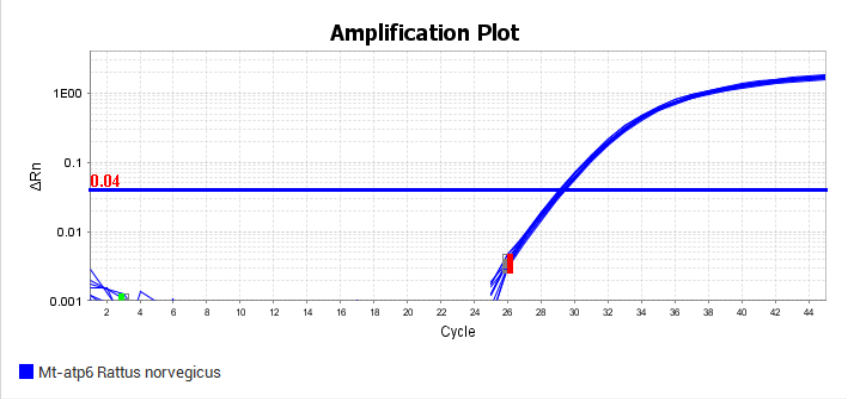

Figure 2. Amplification curve *Mt-atp6 Rattus norvegicus* gene using TaqMan probe

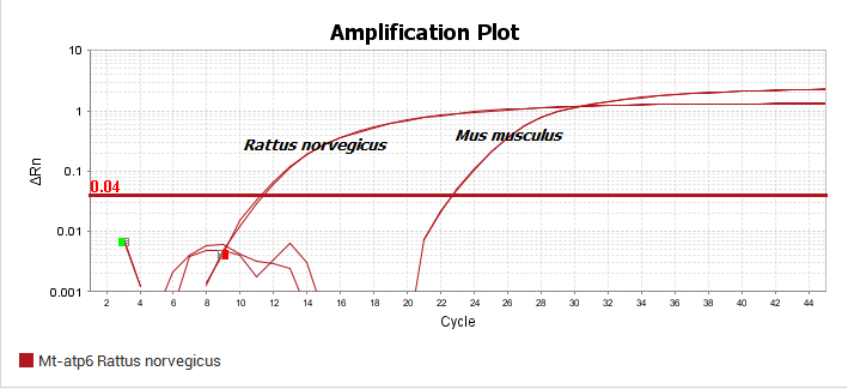

Figure 3. Amplification curve using SYBR Green

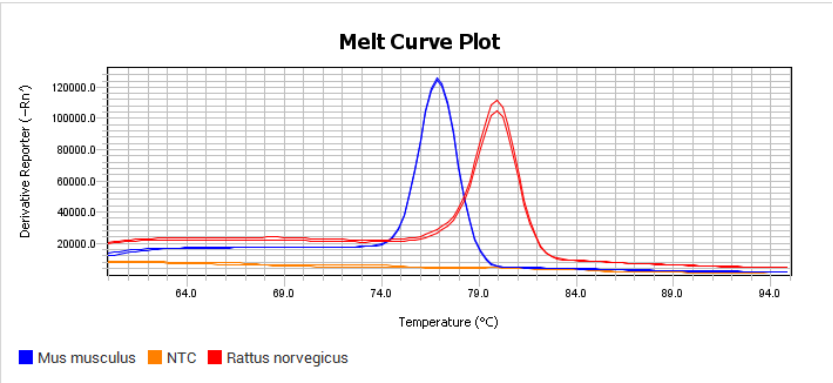

Figure 4. Melt curve *Rattus norvegicus* and *Mus musculus*

From the data contained in Table 5, it can also be seen that the rat meatball sample gave detectable results with Ct values close to each other with an SD of 0.128 because the DNA templates used for amplification were of equal concentration. The Ct value in real-time PCR cycle analysis is influenced by the concentration of the template DNA used [30]. From Table 5, it can also be seen that NTC and non-target samples (chicken, squid, tuna, guinea pigs, and mice) showed no detectable results. The absence of amplification in NTC indicates that in the testing process carried out there was no contamination of the target DNA with the reagents used for the amplification process. In the non-target sample used, the amplification process also did not occur.

Table 5. Ct value using TaqMan probe

Sample	Ct	Ct SD
Rat meatball 1	29.393	0.128
Rat meatball 2	29.197	
Rat meatball 3	29.348	
Rat meatball 4	29.485	
Rat meatball 5	29.127	
Rat meatball 6	29.371	
Rat meatball 7	29.145	
Rat meatball 8	29.453	
Rat meatball 9	29.204	
Rat meatball 10	29.299	
Negative control	Not detected	
Negative control	Not detected	
NTC	Not detected	
NTC	Not detected	

The expected results of the PCR assay using SYBR green were the same as the results of the PCR assay using the TaqMan probe, where the PCR assay can only amplify the *Mt-atp6 Rattus norvegicus* gene (from positive samples). However, the results obtained with the negative control (*Mus musculus*) used in this assay were amplified as well. This is shown by the amplification

curve produced by the positive sample at Ct 11.4 and the negative control at Ct 22.7 (Figure 3) and the melt curve that forms a peak in the positive sample with Tm 79.9°C and negative control with Tm 76.8° C (Figure 4).

The amplification carried out using SYBR Green was able to detect the *Mt-atp6 Rattus norvegicus* gene with good effectiveness. However, SYBR Green has its limitations. Figure 3 shows that *Mus musculus* can also be amplified. This happens because SYBR Green is a dye that will intercalate with double-stranded DNA. In the amplification process, SYBR Green will fluoresce every time it attaches to DNA. So it takes a primer pair that is very specific to only one target species when amplifying using this chemistry [31]. *Mus musculus* and *Rattus norvegicus* are closely related. They come from the same taxonomy family, the Muridae. The orthologous gene pairs distribution was related to the genetic relationship. The species in the same family shared the largest number of orthologous gene pairs [32].

Rahayu et al., in their research also found that determination of the presence of pork DNA in meat samples using melting curve analysis produced no difference between beef or pork or a mixture of pork [33]. In a previous study, Dooley et al. used the same pork Cyt b primer in the TaqMan probe real-time PCR assay and only specifically amplified pork until the cut-off cycle [34].

The melt curve in Figure 4 consists of 1 peak. This indicates that in the amplification process there are no non-specific amplification products. The use of SYBR Green makes it possible to perform melt curve analysis. Good amplification results should produce a single peak. The presence of additional peaks usually indicates that there is a non-specific amplification product [35].

The sequencing results of amplicons from meatball samples containing rat meat (Figure 5) confirmed that the primer used specifically amplifed the *Mt-atp6 Rattus norvegicus* gene. The sequencing of the amplicons matched the target DNA sequences (*Rattus norvegicus*). The results of the nucleotide BLAST analysis showed that the amplicons only recognized the DNA sequence of *Rattus norvegicus* with percent identity 100%, query cover 99%, and E-value $3x10^{-77}$. Alignment using Nucleotide-BLAST makes it possible to perform sequence similarity searches against several databases contained in the NCBI GenBank. The level of similarity of nucleotide sequences is good if the Query Cover is 100%, percent identity gives 100% results, and the E-value ≤ 0.01 [26].

Figure 5. Sequencing results of positive sample

Primer and probe pair that was successfully designed in this study can be used to specifically detect rat meat. However, if the amplification is carried out using SYBR Green so that the use of the probe is omitted, other species that are closely related to *Rattus norvegicus* can then be amplified as well.

4. Conclusions

The primer and probe pair for detecting rat meat was designed with an amplicon size of 161 bp. In silico study and empirical validation with experiments in the laboratory proved that the primer and probe pair used were able to amplify the *Mt-atp6 Rattus norvegicus* gene specifically. We recommend full validation of the method using this primer and probe pair so that it can be used as a valid test method.

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