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Detection of Rat Meat Adulteration in Meat Ball Formulations Employing Real Time PCR

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ABSTRACT

Rat meat is not halal for Muslims, so that the presence of rat meat in any food is a crucial issue. The aim of this study was to design specific primer from Mitochondrial Cty b *Rattus argentiventer* that can be used for determining rat meat contamination or rat meat adulteration in meatball formulation using, Real Time Polymerase Chain Reaction (RT-PCR). The specificity of primers was confirmed in fresh tissue from pigs, cows, chickens, goats, rabbits and white mice. The designed primers were then used to analyze rat meat DNA in meatball formulation made from rat meat and beef mixture at 1, 2, 3, 5, 10, 25, 50 and 100% incorporation of rat meat. The repeatability test was performed by measuring the amplification from fresh rat tissue and rat meat in meatball. Primers were also subjected to sensitivity test of 6 dilution series (50000, 5000, 500, 50, 5 and 0.5 pg μL^{-1}) of rat tissue. From two primers designed, primers cytb 42 (forward: 5'-TAA CCA CTC CTT CAT CGA CCT T-3'; reverse: 5'-CCC CGT TGG CGT GTA AAT A-3') were more specific to evaluate the presence of rat meat in fresh tissue and in meatball formulation at optimum annealing temperature of 61.4°C. The primers can be used for DNA identification by RT-PCR with sensitivity expressed by limit of detection of 5 pg or in meatball formulation at concentration of 1% rat meat.

Key words: Rat meat, meatball, mitochondrial cytb, real-time PCR

INTRODUCTION

In recent years, Indonesian society is disturbed by the issue of beef meatballs mixed with rat meat. Due to the higher price of beef, many sellers took the initiative to replace beef meatballs with rat's meat, which is much cheaper than beef. The public unrest occurs, because rat's meat is not halal for Muslims (JAKIM., 2004). Besides, most people assume that the rats are animals that are not suitable for consumption. Besides the aspects of halal meat for Muslims, rats can also cause many medical problems for humans, since, they are carriers or cause some deadly diseases, such as; Pes, Salmonellosis and Leptospirosis (Alfin, 2012). Based on these issues, it is necessary to study physico-chemistry and molecular biology to detect, whether the food products are contaminated with rat's meat or not.

Meatball is a kind of popular food that can be derived from beef, chicken, or pork (Purnomo and Rahardiyan, 2008). The replacement of beef meatballs with rat's meat can be categorized, as adulteration (Rahmania *et al*., 2015). Numerous analytical techniques have been developed for analyzing rat's meatball, such as; Fourier transform infrared spectroscopy combined with several

chemometrics technique (Rahmania *et al*., 2015), conventional Polymerase Chain Reaction (PCR) (Faizah, 2013) and real time PCR (Hanuraga, 2014; Ningtyas, 2014), using primers from Balakirev and Rozhnov (2012).

The objective of this study was to develop real time PCR (RT-PCR) method that can be used in analysis of rat meat in meatball formulation. The use of RT-PCR method is simpler than conventional PCR due to the amplification product can be directly observed and analyzed quantitatively in RT-PCR using the DNA dye or fluorescence probe. Primer specificity is an important criteria in PCR technique, so that the design of specific primer will determine the success of analysis using PCR (Sudjadi, 2008). In this study, two primer pairs designed using Primer NCBI-BLAST software at NCBI website were obtained. Primers designed were subjected to BLAST analysis in order to confirm the specificity of rat's DNA. Two primer pairs used were primer cytb 42 (forward: 5'-TAA CCA CTC CTT CAT CGA CCT T-3'; reverse: 5'-CCC CGT TGG CGT GTA AAT A-3') and primer cytb 63 (forward: 5'-TCC TGC CCC ATC TAA TAT CTC CT-3'; reverse: 5'-GCC CCG TTG GCG TGT AAA T-3'). The most specific primers are used to perform amplification using RTPCR on rat meatball formulation. The primers are also used to analyze commercial beef meatball to identify the presence of rat meat.

MATERIALS AND METHODS

Sample collection and preparation of meatball formulation: The meat of rat (*Rattus argentiventer*) was obtained from farmer's land in Yogyakarta, Indonesia. The white mouse (*Rattus norvegicus*) was obtained from Laboratory of Pharmacology, Faculty of Pharmacy UGM Yogyakarta, Indonesia, while beef, chicken, rabbit and goat as well as, commercial beef meatballs were purchased from several local markets and supermarkets in Yogyakarta Indonesia. All the collected samples were stored at -20°C for further processing and for the preparation of meatball formulation. Meatballs were prepared according to Purnomo and Rahardiyan (2008). Meatball was prepared by emulsifying 90% of fine ground meat (beef and or rat meat) with 10% of starch and mixed vigorously with certain ingredients. The homogenous meat mixture was shaped into balls and then cooked in boiling water for 10-15 min.

DNA isolation: Isolation of DNA from fresh tissue and meatballs formulation was performed by DNA isolationKitK280-50 from Biovision (USA).

PCR amplification: In order to identify and quantify rat DNA in fresh tissue or meatball formulation, DNA isolates were analyzed by real-time PCR with SYBR green® universal PCR master mix fluorescent dye using designed primers at optimum annealing temperature. For each reaction, a total of 20 μL of the above mixture was dispensed (containing of 10 μL SYBR Green master mix, 1 μL primer forward and 1 μL primer reverse, 1 μL 50 ng DNA template and water free RNA-ase). The Amplification was performed with a real-time PCR using PCR CFX96 (Biorad, USA). The thermal cycler protocol was as follows: initial denaturation at 95°C for 15 sec to denature the DNA template completely, annealing and extension at 72°C for 10 sec, the amplification cycles are 30 and the melting curve was performed at 65-95°C using slope 0.5°C/2 sec.

Determination of the specificity, linearity, sensitivity and repeatability of the assay: The selected primers at optimum annealing temperature were applied to RT PCR analysis. The determination of specificity assay was measured using template of DNA from rat, chicken, beef,

rabbit, goat and white mouse. The amplification of rat meatball formulation from rat and beef mixture was 1, 2, 3, 5, 10, 25, 50 and 100% level of concentration and used to determine linearity test. The sensitivity was taken as, being the lowest amount that could be amplified using amplification of 6 dilution series from fresh rat tissue. Repeatability of the assay was measured using 6 times of amplification of DNA isolated from meatball formulation with concentration of 100% rat meat.

Analysis of commercial beef meatball samples: The commercial beef meatball samples obtained from several markets in Yogyakarta, Indonesia, was analyzed, using selected primer and protocol set out above.

RESULTS AND DISCUSSION

The selected primer having G or C bases in the last 5 position of the 3' end less than 3 can increase the specific binding at the 3' (Van Pelt-Verkuil *et al*., 2008). Besides, it does not form GC clamp folds. The length of amplicon which is less than 250 bp can increase the method efficiency (Wang and Seed, 2006). Both pairs of primer were optimized to obtain the appropriate annealing temperature at various gradient temperatures (52.0, 52.7, 54.0, 55.9, 58.4, 60.3, 61.4 and 62.0°C). The primer cytb 42 showed higher intensity than primer cytb 63 (Fig. 1), which is the maximum intensity and the lowest Cq of amplification at temperature of 61.4°C (Fig. 2). Rat DNA has been amplified using primer cytb 42 to check specificity with annealing temperature at 61.4°C and the number of cycles is limited to 30. Rat's DNA showed amplification response at cycles of 19.65 with intensity of 1492 RFU, while bovine DNA, chicken DNA, pig DNA, rabbit DNA, white mouse DNA and goat DNA did not show any amplification at this condition (Fig. 3).

The amplification of rat meatball formulation from rat and beef mixture at 1, 2, 3, 5, 10, 25, 50 and 100% concentration was also performed with same RT PCR protocol as describe before. Standard curve showed coefficient of determination (R^2) of 0.983, slope -4.136 and y-intercept of 28.916. These values meet criteria of linearity curve, while the amplification efficiency at 74.5% exceeds the criteria in Bio-Rad (2006), namely 90-110%. The low efficiency values can be caused by lack of pipetting precision and DNA extraction methods (Muhammed *et al*., 2015).

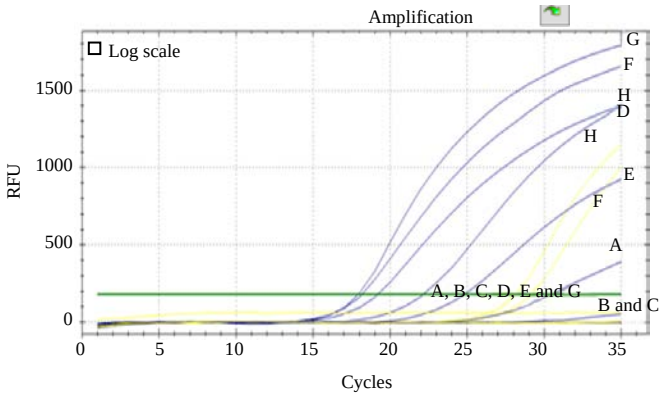

Fig. 1: Amplification curve using primer cytb 42 (blue) and primer cytb 63 (yellow) at various annealing temperature at A: 52.0, B: 52.7, C: 54.0, D: 55.9, E: 58.4, F: 60.3, G: 61.4 and H: 62.0°C

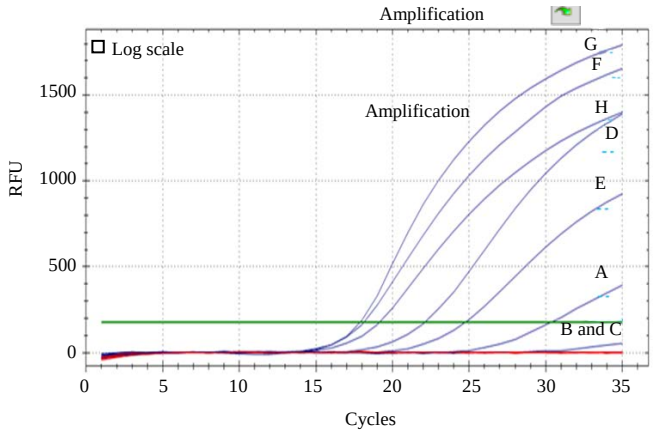

Fig. 2: Amplification curve using primer cytb 42 at various various annealing temperature at, A: 52.0, B: 52.7, C: 54.0, D: 55.9, E: 58.4, F: 60.3, G: 61.4 and H: 62.0ºC

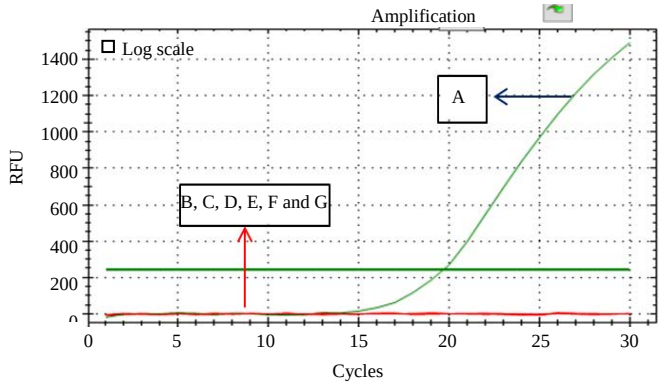

Fig. 3: Amplification curve using isolated DNA of A: Rat, B: Bovine, C: Pig, D: Goat, E: Rabbit, F: Chicken and G: White mouse

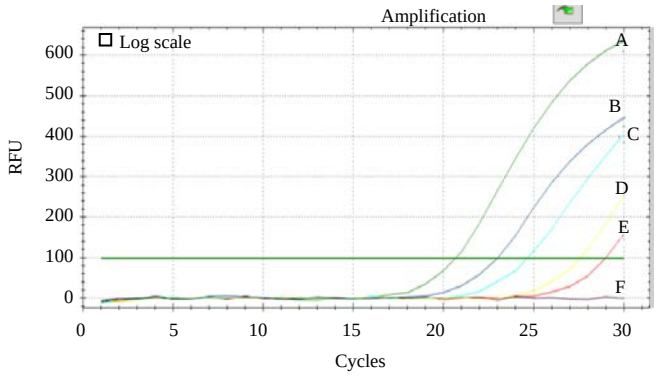

Fig. 4: Amplification curve of 6 dilution series A: 50000, B: 5000, C: 500, D: 50, E: 5 and F: 0.5 pg μL^{-1}, using isolate DNA from fresh rat tissue

Method sensitivity was expressed as Limit of Detection (LoD), determined by amplification of 6 dilution series (50000, 5000, 500, 50, 5 and 0.5 pg μL^{-1}). Rat DNA from fresh tissue can still be amplified up to 5 pg while at 0.5 pg, there is no amplification response up to 30 cycles (Fig. 4).

Repeatability tests were also performed on meatball formulation with concentration 100% rat meat and also fresh rat tissue in order to confirm the consistency of PCR result. CAC (2010) requires that the Coefficient of Variation (CV) is less than 25%. The repeatability test demonstrated that the amplification occurs in all sample (Fig. 5) with CV of rat meatball is 1.91% and CV of fresh rat tissue is 1.01%.

The primer cytb 42 along with real-time PCR analysis protocol (annealing temperature at 58.4ºC and 30 cycles amplification), was then applied to identify the presence of rat DNA in commercial beef meatball. The result showed that no amplification responses occur in all commercial beef meatballs, indicating that there was no rat meat contamination in evaluated meatballs (Fig. 6).

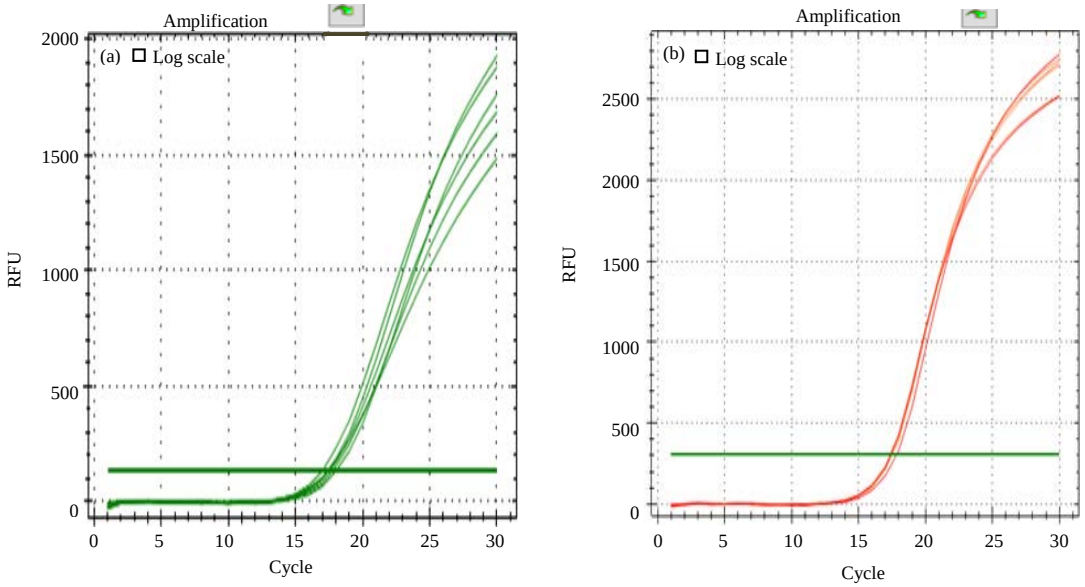

Fig. 5(a-b): Amplification curve of meatball formulation with concentration 100% rat meat (green) and fresh rat tissue (red)

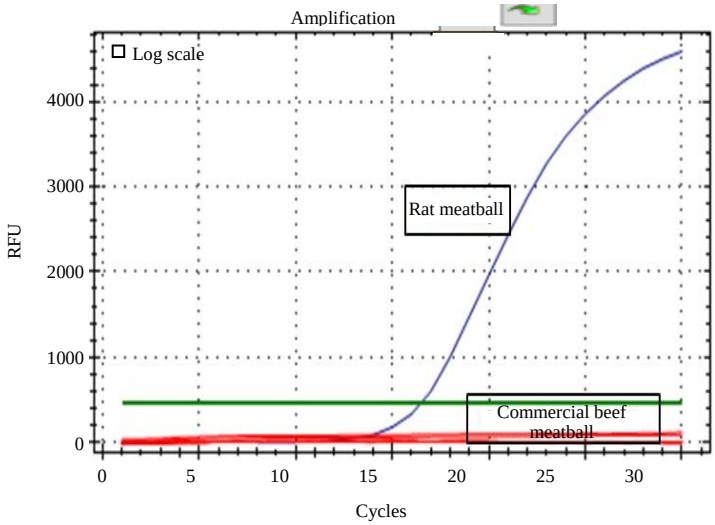

Fig. 6: Amplification curve of commercial beef meat ball samples (red) and rat meatball, using primer cytb 42

CONCLUSION

The primer cytb 42 is more specific to be used for identification of rat meat in fresh tissue and meatball formulation at optimum annealing temperature of 61.4°C. The primer can be used for DNA identification by RT-PCR with sensitivity at 5 pg or in a mixture of rat and beef meatballs formulation at concentration of 1% rat meat. All commercial beef meatballs did not contain rat DNA.

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