Mitochondrial ND-1 gene-specific primer polymerase chain reaction to determine mice contamination in meatball

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Abstract

A specificity method to detect mice meat contamination in beef meatballs using specific primer-polymerase chain reaction (PCR) technique has been developed. The primer ND1-P1 primers were designed using primer-BLAST software using mtDNA of mice as a template. The Primer ND1-P1 forward (5’-CGGCATCCTACAACCATTTGC-3’) and reverse (5’-CGGCTCGTAAAGCTCCGAA-3’) was able to amplify a 294 bp fragment of ND1 gene in mice mtDNA. The primers have been proven precise with only amplify the target fragment in mice meatball but not in another meatball including beef meatball, chicken meatball, pork meatball, horse meatball, and goat meatball. The present of mice meat in meatballs can be detected at a concentration as low as 5% (w/w). The ND1-P1 primer is potentially used as a specific marker for detection of mice meat in the meat products.

Introduction

Authentication of animal species present in food is essential according to Islam law, which states that Moslem have to consume only Halal food. Pork and meat from any wild animal are prohibited to be consumed. However due to the price difference to beef, pork has usually been used in meat based food forgery. In Indonesia, lately counterfeiting meatball is also performed with other wild animal meats such as mice. A simple, fast, cheap and selective method to identify the present of mice in the meatball should be developed to help fight this fraud.

Identification adulterated meat based product have been developed including ELISA (Asensio et al., 2008) FTIR spectrometry (Rohman et al., 2011; Rahmania et al., 2015) as well as HPLC (Giaretta et al., 2013) and LC-MS/MS (Sarah et al., 2016). DNA-based methods offer advantages in accuracy, simple and can be applied to the processed food due to the stability of the DNA. Various DNA method have been reported to identify porcine including species specific PCR (Karabasanavar et al., 2014); PCR-RFLP (Ali et al., 2011; Raharjo et al., 2012), PCR-RAPD (Farouk et al., 2006), real-time-PCR (Rahmawati et al., 2016; Maryam et al., 2016) and DNA barcoding ( Di Pinto et al., 2013).

Several methods on mice adulteration have been reported. Steube et al. (2008) have conducted development of an assay to detect murine and related species by means of a regular PCR. However, the method is shown cross-reactivity with other species (Sun et al., 2012). Real-time PCR using Taqman probe have been established including simplex and multiplex PCR (Ali et al., 2015, Fang and Zhan, 2016). Real-time PCR seems key advantages on specificity but developing specific primer standard PCR to detect mice adulteration remain challenging as this technique quick and straightforward method that can be quickly developed to Real-time PCR. However, regular specific primer PCR method offers benefit in term of cost of analysis.

Most of the reported DNA method to identify species use mitochondrial DNA (mtDNA) due to its high variety of the sequence among species due to high rate mutation of mtDNA. Since mtDNA has a high copy number (more than 100 per cell), so it can be used for analysis of the little amount of sample. The sequence of CytB and D-loop are very famous for designing primer of the probe for Taqman (Fang and Zhan, 2015) as well primer for specific primer PCR (Ali et al., 2015) and PCR-RFLP (Raharjo et al., 2012). However, other genes present in mtDNA have the same possibility to be a candidate for primer or probe designing for species identification. This study reported PCR specific primer using new primers design based on another part of mtDNA including the 12SrRNA gene and the ND1-gene of mice (Mus...
Materials and Methods

Primer and samples

The mitochondrial DNA (mtDNA) sequence of Mus musculus castaneus (NCBI Access Number: AB042432) was used to design the primer. Laboratory prepared of mice meatball, and beef meatball was used as positive control negative control, respectively. Laboratory prepared of beef meatball, chicken meatball, pork meatball, horseflesh meatball and goat meat were used in specificity test. Beef meatball with various content of mice meat (1, 2, 5, 10, 25, 50 and 75% (w/w) were employed in sensitivity test. The commercial meatball was purchased at a local supermarket in Yogyakarta. (Patria, 2014).

DNA isolation and PCR

The DNA isolation began by grinding the meatball into powder followed by steps according to Sambrook isolation procedures with slight modifications (Sambrook et al., 1989). Amplification of specific DNA fragment was performed in a total volume of 25 µL containing 1 x PCR reaction buffer, one µg of isolated DNA accompanied by ten pmol of each primer to Ready-to-go PCR beads tube and DNAse-free water. The PCR was run for 30 step cycles. The temperature program of each cycle was denaturation at 95°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min. An initial denaturation step at 95°C for 5 min and a final extension at 72°C for 5 minutes were employed before and after the cycle respectively. Electrophoretic separation of 10 µL of PCR products was performed in 2% agarose gel in 1 x TBE buffer, pH 8.0 at constant voltage (100 volts) for 45 min.

Specificity test and cut off determination

The specific nature of the primers was investigated by performing amplification of mtDNA isolated from meatball of mice meat and other tested species. Among tested meatball were a chicken meatball, pork meatball, horse meatball, goat meatball and cattle meatballs. The cut off of the method represent the limit detection of the method was determined by testing series of beef meatballs with various concentration of mice meat contamination (1, 2, 5, 10, 25, 50% (w/w)). The lowest level of rat contamination in meatballs which still give positive result obtained as the cut off of the methods. The method was then used to verify five sample commercial meatball that sold at supermarkets in Yogyakarta (Patria, 2014).

Result and Discussion

The primer design using mice mtDNA produced nine primer pairs with each of the three pairs of primer amplify 12SrRNA gene, ND-1 gene, and D-loop region. Although the specificity of the primers has been calculated by software, the primers were then screened further to select best designed to be synthesized. The primer sequence was aligned to mtDNA of the species which used as species check during primer design. High homology between primers to the sequence could lead amplification this mtDNA by the primers which mean the primers were not unique to mice mtDNA. There were three primer pairs as most probable mice specific primers as shown in Table 1 that were synthesized and further used in the experiment.

Among three candidate of the area as amplification target, only D-loop is well known for species identification due to its intra-species variety. Genes encode NADH dehydrogenase subunit 1 (ND1), and 12SrRNA have never been reported before. Some previous studies reported the success of the animal species identification using primers amplified NADH dehydrogenase subunit 5 (ND5) and ATPase subunit 6 (ATP6) (da Fonseca et al., 2008; Kitpipit et al., 2014). Meanwhile due to the clear evolutionary patterns have made cytochrome b (cytb) gene another target for specific primers (Xin et al., 2006).

Beef meatball is the primary object of mice meatball adulteration. Therefore, the mice specific primer was tested for specificity to the beef meatball prior meatball of other meat species. The results of electrophoresis analysis of PCR of beef and mice meatball mtDNA using three primers are shown in Figure 1. All three pair primers gave PCR amplification with the size as expected, 133 bp of 12SrRNA-P3, 207 of D-loop-P2 and 294 bp of ND1-P1 respectively. However primers D-loop-P2 gave other PCR product with size approximately 550 bp. It seems that D-loop-P2 has more than one site of annealing leading to a non-specific product.
Amplification of beef meatball as negative control gave no amplification result as expected for D-loop-P2 and ND1-P1 but 12SrRNA-P3 gave amplification product with the same size as mice meatball sample. ND1-P1 and D-loop-P2 could be candidates for mice specific primers. A similar result to D-loop-P2 primer was previously reported by Maryam et al. (2016). RT-PCR of a different D-loop primer resulted in two amplification fragment. However since no amplification observed during amplification of other species, the primer could still claim as pork specific primer.

The annealing temperature is a critical parameter in order the increased specificity of a primer. Typically increasing the annealing temperature is one way that should be performed to improve the specificity. In the case of 12S rRNA primer, various annealing temperatures have been applied. The band of beef amplification was reduced with increasing of annealing temperature but not too significant (data not shown). It might be at a certain high temperature of annealing the 12SrRNA primer would become mice specifically, but the binding of the primer to mice mtDNA would be weak leading to a higher concentration of mice mtDNA needed to give a positive result. It means that persuade specificity by increasing annealing could cost sensitivity of the method. In the case of D-loop primers the non-specificity of the primer could be caused by appearing in a series of four polypyrimidine in a primer sequence (forward primer 5’TATCGCCCATACGTTCCCCT3’ and reverse primer 5’AGGTGATTTGATTTGCGGA3’). Polypyrimidine and polypurine cause the primer adheres at another template out of target region. The result corresponds with the theoretical reported by Erlich (1989). However, since the non-specific amplification by both D-loop and 12SrRNA primer prove that although the design process has been set to be mice specific and followed by further screening using homology analysis, the selected primer did not give sophisticated result in PCR experiment.

The only primer show specificity to mice when tested with beef meatball was the ND1-P1 primer. Further specificity analysis of this primer to other species was performed. Figure 2 demonstrate the result of amplification of mtDNA isolated from meatball of various meat using the ND1-P1 primer. It concludes that the ND1-P1 primer is a mice specific primer since the primers only give a positive amplification product of 294 bp from mtDNA of mice meatballs and no amplification of other species meatball. This data also lead to the possibility of the primers to be used to detect the present of mice meat in the mixture of commonly consumed meat (beef, chicken, pig, horse, and goat).

The cut off determination was aimed to check how low the level of mice content in the meatball can be detected. The cut off was tested on the beef meatballs mixed with mice meat with various level of concentration (1, 2, 5, 10, 25 and 50% (w/w)). The electrophoresis analysis of the cut-off determination was shown in Figure 3. It was observed that the mice
specific primer capable of detecting the present of 5% contamination of mice meat in the beef meatball. The much lower limit of detection data was reported using RT-PCR such as mice detection up to 0.1% in the meat mixture Fang and Yang (2016) or 1% in meatball (Ali et al. 2015). However, the value of the cut-off was quite small and comply with the requirement since the method was purposed to check food adulteration. The reason of using mice meat is usually commercial, to replace more expensive beef meat, therefore the amount of mice meat added to the meatball recipe must be much higher than 5%. The similar results of cut off was reported by Raharjo et al. (2012) in the analysis of pork meatball adulteration using PCR-RFLP. This PCR-RFLP report is more comparable to this current study since both using regular PCR. The meatballs are typically made by mixing raw materials in a solid form that makes the meatballs possess of a less homogeneity thus inhibiting detecting the presence of adulterated mixture with concentration under 5%.

The specific primer-PCR method applies for meatballs product from the various market. Six samples of meatballs from the different market are tested for rat contamination by using the ND1-P1 primer. Electrophoresis visualization of PCR product shows that no interference in six samples meatballs are represented at Figure. 4. This result can be a source of discourse that specific primer-PCR is very useful for routine analysis of monitoring meat products being sensitive and quick analysis.

Conclusion

The ND1-Primer designed by primer-BLAST which derived from Mus musculus c. Mitochondrial DNA could be correctly used for detect rat contamination in meatballs. A specific primer-PCR technique using ND1-P1 Primer is sensitively detected up to 5% level of contamination. This technique is useful and looks like a promising method for detection of mice meat in meatball products for halal authentication.

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