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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'-CGGCTCGTAAAGC-TCCGAA-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*

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musculus) mtDNA.

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.

Primer design

The primers have been developed using primer-BLAST based on *Mus musculus castaneus* mtDNA. The designed primer was checked their specificity toward mtDNA sequences of cow *(Bos taurus)* (AY526085), pork *(Sus scrofa)* (AF034253), chicken *(Gallus gallus)* (X52392), horse *(Equus caballus)* (X79547) and goat *(Capra aegagrus)* (KT290893). The targeted primers were set for 19-21 base length, with %GC 50-65% and targeted to amplify 100-300 bp mtDNA fragment (Nuryanti, 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.

Table 1. The candidate of mice specific PCR primers

Primer code	Sequence (5'.3')	Size of amplification (bp)
12S rRNA-P3	F: CATGCAAACCTCCATAGACCG R: TTATCACTGCTGAGTCCCGT	133
D-loop-P2	F : TATCGCCCATACGTTCCCCT R : AGGTGATTGGGTTTTGCGGA	207
ND1-P1	F: CGGCATCCTACAACCATTTGC R: CGGCTCGTAAAGCTCCGAA	294

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 12R-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 12RrRNA 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'AGGTGATTGGGTTTTGCGGA3'). 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

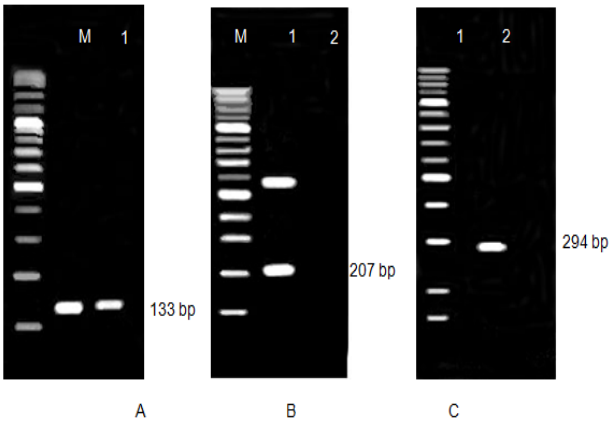

Figure 1. Electrophoresis of PCR product of meatball using different primers (A) 12S rRNA-P3 primer, (B) D-loop-P2 primer and (C) ND1-P1 primer. (M) DNA marker, (1) mice meatball (positive control), (2) beef meatball (negative control)

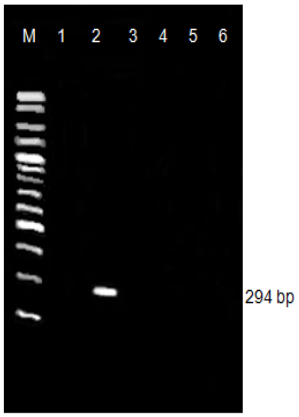

Figure 2. Electrophoresis of PCR product of meatball made of various meat. PCR amplification using ND1-P1 primer. (M) DNA marker, (1) beef meatball (negative control), (2) mice meatball (positive control), (3) chicken meatball, (4) pork meatball, (5) horse meatball, (6) goat meatball

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

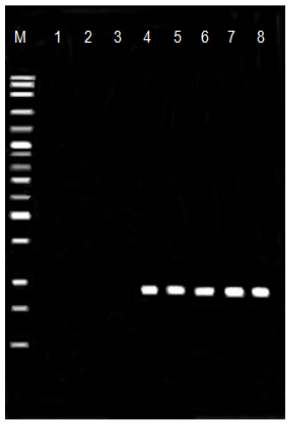

Figure 3. Electrophoresis of PCR product of meatball with different level of mice concentration. PCR amplification using ND1-P1 primer. (M) DNA marker, (1) beef 100% meatball (negative control) , (2) mice 1% : beef 99% meatball, (3) mice 2% : beef 98% meatball, (4) mice 5% : beef 95% meatball, (5) mice 10% : beef 90% meatball, (6) mice 25% : beef 75% meatball, (7) mice 50% : beef 50% meatball and (8) mice 100% meatball (positive control)

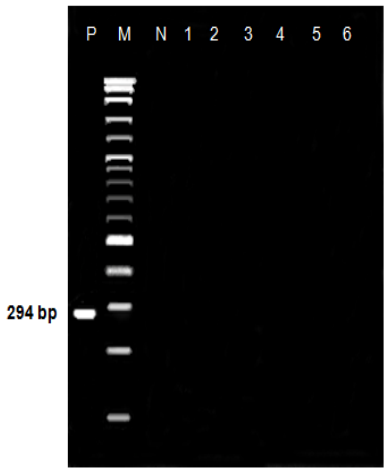

Figure 4. Electrophoresis of PCR product of meatballs from the various market. PCR amplification using ND1-P1 primer. (M) DNA marker, (N) negative control, (P) positive control, (1 and 2) roving meatballs, (3 and 4) supermarket, and (5 and 6) traditional market

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-RPLP 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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References

Ali, M.E., Razzak, M.A., Hamid, S.B.A., Rahman, M.M., Al Amin, M., Rashid, N.R.A. and Asing. 2015. Analytical Methods: Multiplex PCR assay for the detection of five meat species forbidden in Islamic foods. Food Chemistry 177: 214–224.

Ali, M.E., Hashim, U., Mustafa, S. and Man, Y.B.C. 2011. Swine-specific PCR-RFLP assay targeting mitochondrial cytochrome b gene for semiquantitative detection of pork in commercial meat product. Food Analytical Methods 5 (3):613-623.

Asensio, L., González, I., García, T. and Martín, R. 2008. Review: Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). Food Control 19 (1): 1-8.

da Fonseca, R. R., Johnson, W. E., O'Brien, S. J., Ramos, M. J. and Antunes, A. 2008. The adaptive evolution of the mammalian mitochondrial genome. BMC Genomics 9: 119.

Di Pinto, A., Di Pinto, P., Terio, V., Bozzo, G., Bonerba, E., Ceci, E. and Tantillo, G. 2013. DNA barcoding for detecting market substitution in salted cod fillets and battered cod chunks. Food Chemistry 141 (3): 1757-1762.

Erlich, H. A. 1989. PCR Technology : Principles and Amplifications for DNA Amplification. London: Palgrave Macmillan.

Fang, X. and Zhan, C. 2016. Detection of adulterated murine components in meat products by TaqMan© real-time PCR. Food Chemistry 192: 485-490.

Fajardo V., González I., López-Calleja I., Martín I., Rojas M., Hernández P.E., García T. and Martín R. 2007. Identification of meats from red deer *(Cervus elaphus),* fallow deer *(Dama dama),* and roe deer *(Capreolus capreolus)* using polymerase chain reaction targeting specific sequences from the mitochondrial 12S rRNA gene. Meat Science 76(2): 234-40.

Farouk, A., Batcha, M. F., Greiner, R., Salleh, H. M, Salleh, M. R. and Sirajudin, A. R. 2006. The use of a molecular technique for the detection of porcine ingredients in the Malaysian food market. Saudi Medical Journal 27 (9): 1397-1400.

Giaretta, N., Di Giuseppe,A.M.A., Lippert, M., Parente, A. and Di Maro,A. 2013. Myoglobin as marker in meat adulteration: A UPLC method for determining the presence of pork meat in raw beef burger. Food Chemistry 141 (3): 1814-1820.

Karabasanavar, N.S., Singh, S.P., Kumar, D. and Shebannavar, S.N., 2014. Detection of pork adulteration by highly-specific PCR assay of mitochondrial D-loop. Food Chemistry 145: 530-534.

Kitpipit, T., Sittichan, K. and Thanakiatkrai, P. 2014. Direct-multiplex PCR assay for meat species identification in food products. Food Chemistry 163: 77–82.

Maryam, S., Sismindari, S., Raharjo, T.J., Sudjadi and Rohman, A. 2016. Determination of Porcine Contamination in Laboratory Prepared dendeng Using Mitochondrial D-Loop686 and cyt b Gene Primers by Real Time Polymerase Chain Reaction. International Journal of Food Properties 19 (1): 187-195.

Nuryanti, I. 2014. Specificity testing of detection method of rat meat contamination in beef meatballs using specific primer-polymerase chain reaction (PCR) technique based on mitochondrial DNA sequence. Thesis. Department of Chemistry, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Patria, F.P. 2014. Analysis of rat meat content in meatballs by using polymerase chain reaction (PCR) ppecific primer ND1 gene mitochondrial DNA. Undergraduate Thesis, Department of Chemistry, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Rahmania, H., Sudjadi and Rohman, A. 2015. The employment of FTIR spectroscopy in combination with chemometrics for analysis of rat meat in meatball formulation. Meat Science 100: 301-305.

Rahmawati, Sismindari, Raharjo, T.J., Sudjadi and Rohman, A. 2016. Analysis of pork contamination in Abon using mitochondrial D-Loop22 primers using real-time polymerase chain reaction method. International Food Research Journal 23 (1): 370-374.

Raharjo, T.J., Cahyaningtyas, W., Surajiman, Istini and Pranowo, D. 2012. Document Validation of PCR-RFLP testing method to detect porcine contamination in chicken nugget. Indonesian Journal of Chemistry 12 (3): 302-307.

Raharjo, T.J., Alfiraza, E.N., Enjelina, E., Pranowo, D. 2017. Validation of a non-specific dye real-time PCR assay for porcine adulteration in meatball using ND5 primer. Indonesian Journal of Chemistry 17 (2): 167 - 174

Rohman, A., Sismindari, Erwanto, Y. and Che Man, Y. B. 2011. Analysis of pork adulteration in beef meatball using Fourier transform infrared (FTIR) spectroscopy. Meat Science 88 (1): 91-95.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

Sarah, S.A., Faradalila, W.N., Salwani, M.S., Amin, I., Karsani, S.A. and Sazili, A.Q. 2016. LC–QTOF-MS identification of porcine-specific peptide in heat treated pork identifies candidate markers for meat species determination. Food Chemistry 199: 157-164.

Steube, K.G., Koelz, A.L. and Drexler, H.G. 2008. Identification and verification of rodent cell lines by polymerase chain reaction. Cytotechnology 56:49–56.

Sun, W., Li, G., Liu, H., Zhong, W., Zhang, H., Bao, K., Xu, C., Yang, Y. and Wang, Z. 2012. Detection of mink *(Mustela uison)* DNA in meat products using polymerase chain reaction PCR assay. Asian Journal of Animal and Veterinary Advances 7 (12): 1348-1355.

Xin, W., Yue-Hui, M. A. and Hong, C. 2006. Analysis of the genetic diversity and the phylogenetic evolution of chinese sheep based on Cyt b gene sequences. Acta Genetica Sinica 33(12): 1081–1086