Detection of Rat (*Rattus Norvegicus*) DNA Fragments using Specific: Species Primer mt-DNA *12S rRNA* and *Cyt-b* with Polymerase Chain Reaction (PCR) Technique

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Abstract: A pair of species-specific mt-DNA primer 12S rRNA has been designed based on rat (Rattus norvegicus) DNA sequences. In this study, the specificity test of 12S rRNA and Cyt-b were carried out to detect rat DNA fragments. Samples of this research were used non-halal animal meat consisted of rats, lamb, dog, pig and halal animal meat consisted of cow, chicken, and horse. Furthermore, DNA was isolated from animal meat using modification of chloroform isoamyl alcohol method then quantitatively tested for its concentration and purity. Animal meat isolates were amplified using 12S rRNA and Cyt-b primers using PCR techniques. Analysis of PCR results using agarose gel electrophoresis 1.5%. The amplification results showed that 12S rRNA primer produced DNA bands of 228 bp length and Cyt-b primer produced DNA bands of 603 bp length. The amplification results showed that both of 12S rRNA and Cyt-b primers were specific to detect rat DNA fragments. Thus, both of primers are recommended to be further tested for sensitivity and applied to processed meat products such as meatballs, sausages, and corned beef.

1 INTRODUCTION

Rats often cause health issues such as bubonic plague, leptospirosis, murine typhus, and plague (Center for Disease Control and Prevention, 2011). Ironically, rat meat holds a high possibility to be used in various food products, most of the time, meatballs. This adulteration case rises among society in Indonesia as it exhibits promising profit. Moreover, that such adulteration could not be easily identified. The recent case of meat adulteration were 63 tons. Those cases were substituted beef into pig meat that claimed as pure beef (Warta Ekonomi, 2012). This criminal case complicate the Muslims society as they are prohibited from consuming non-halal foods. The lack of food management that related to halal, safety, and health resulting the production process violations (Yasmin, 2013). The low awareness of the importance about halal logo, halal certification at Slaughterhouses (RPH) and Poultry Slaughterhouses (RPU), also the low the protection from local governments leads the increasing of new cases arise (Arifiani, 2019). Indonesian Law no. 33 year 2014 regarding to Halal Product Guarantee is regulated to eliminate the meat adulteration cases.

The developing of an effective and efficient method in meat contamination detection and adulteration are essential in order to support the guarantee of Halal. *Polymerase Chain Reaction* (PCR) is a method of DNA analysis by amplifying DNA in vitro involving several stages, it's denaturation, annealing, and extension (Handoyo et al., 2000). Some constituent components including DNA template (DNA template); primer; deoxynucleoside triphosphates (dNTPs); DNA polymerase enzyme; and PCR buffer, are required to optimize the process. The primers were analyze as primers are the success key in PCR technique. *Mt-DNA* 12S rRNA gene contains high base variations between species and low base variations in the same species (Kitpipit, 2014). Several advantages of the PCR technique are its specificity and high sensitivity, short time, and its ability to detect contaminated samples and to work on samples with a complex mixture (Aminah et al., 2019). The PCR technique is known as accurate, fast, affordable, and able to

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analyze a DNA (Deoxyribonucleic acid) sample from high to low concentration (Arslan et al., 2006; Aminah et al., 2019).

Specific primers of mt-DNA 12S ribosomal RNA have been developed for detecting and identifying DNA fragments of cats (*Felis catus*), dogs (*Canis familiaris*), and rat (*Rattus norvegicus*) that contained in food and food ingredients. The results showed that 3 pairs of primers that had been designed from the DNA of 24 animals and plants produced specific sequences with lengths of 108 bp, 101 bp, and 96 bp were successfully amplified in cats, dogs, and rat (Martin et al., 2014). The comparison primer was mt-DNA Cyt-b which had been tested for it's specificity on detected DNA fragments of rat (Nuraini et al., 2012). Cyt-b primers that designed manually, had used to amplify 6 species which are goats, chickens, cows, lamb, pigs, and horses successfully, while the Cyt-b primers that designed using software based on the Cyt-b sequence on *Rattus norvegicus* were success to amplify 7 animal species which indicated that the Cyt-b primer was specific. It is possible to detect tissue samples of cats, dogs, rats on food and foodstuffs in high sensitivity and specificity by using specific primers (Martin et al., 2014). In this study, a species-specific mt-DNA 12S rRNA primer was designed and the specificity was analyzed in detecting DNA fragments in rats as the initial stage of testing. The comparison genes used mt-DNA Cyt-b primers that had been studied previously for the detection of rat DNA fragments. It is expected that further research will be develop in concerning primer testing based on the primer sensitivity and its applicate on detecting rat DNA fragments in various meat products such as meatballs, corned beef, and sausages.

2 RESEARCH METHODS

The samples of non-halal raw meats were collected from rats (positive control), dogs, and pigs while the halal meat samples were obtained from cattle, chicken, horse, and lamb (negative control). The DNA isolation was performed by Chloroform Isoamyl Alcohol method modified from Sambrook and Russel's (1989) procedure. The reagent used in DNA isolation were consisted of STE lysis buffer (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 8), 10% SDS, and 10 mg / mL Proteinase-K. C: I (Chloroform: Isoamyl) with a ratio of 24:1 was used in the purification stage. Absolute ethanol (EtOH) was used in the DNA washing stage, treated on cold condition. In the DNA precipitation stage, 70% ethanol and 5M NaCl were used. The final stage was DNA elution that used TE buffer pH 7.6. The modified procedures were used Pro-K instead of phenol in the DNA purification stage.

The results of DNA isolation were analyzed quantitatively using (Nanodrop / ND-1000 UV / Vis) to obtain the concentration and purity, and were amplified using Thermal Cycler (Applied Biosystem / PCR System 9700) and Thermocycler (Takara / Version 3 Model TP600). The PCR process with a total volume of 10µl consisted of 5 µl Go Taq Green Master Mix (PROMEGA), a pair of 12S rRNA primers with a concentration of 5 pmol / µl as much as 0.5 µl, DNA template 1 µl, and ddH2O 3 µl. The PCR program for primers 12S rRNA consisted of five stages. Firstly, the initial denaturation at 95˚C for 5 minutes. Secondly, denaturation at 95˚C for 30 seconds. Thirdly, the 12S rRNA primers annealing at 54˚C for 45 seconds. Fourth, the extension at 72˚C for 30 seconds, and the last, final extension with a temperature of 72˚C for 5 minutes. The same program was applied for Cyt-b primers amplification, excluding the annealing step 54˚C for 45 seconds.

12S rRNA gene primers were specifically designed from mt-DNA rat (*Rattus norvegicus*) based on the data from GenBank NCBI (National Center for Biotechnology Information). The primers were designed manually by bioinformatics programs such as clustal X and bioedit to align the 12S rRNA gene sequences from several test animals, such as cattle (*Bos indicus*), dogs (*Canis lupus familiaris*), pigs (*Sus scrofa domesticus*), chickens (*Gallus gallus*), horse (*Equus caballus*), and lamb (*Ovis aries*). This study was performed the 12S rRNA gene with a target length of 228 bp employing a primer sequence length of 20 bp, GC content (50%), melting temperature of 60°C, and annealing temperature of 54 °C (Figure 1).

Table 1: Primers 12S rRNA and Cyt-b genes.

Primer	Sequence (5' – 3')	Amplicon (bp)	Base
12S rRNA	Forward: GGA CCT AAG CCC AAT AAC GA	228	20
	Reverse: TTC TAC CTT ACC CCT TCT CG		20
Cyt-b	Forward: GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA	603	38
	Reverse: GAA TGG GAT TTT GTC TGC GTT GGA GTT T		28

The comparison primers used forward SIM primers which were designed based on the Cyt-b gene

sequence from 6 animal species. Forward SIM primers were selected according to sequence missmatch within control species of 3-5 bases for 38 bp. SIM was designed longer than species-specific primers with nucleotides measuring 26-29. DNA fragments were formulated using software for determining primers that were designed based on species-specific areas. Reverse primers for detecting the DNA fragments of rat were designed based on primer design software (http://www.ncbi.nlm.nih.gov/tools/primerblast/indexshtml). Reverse primers for rat DNA fragments detection have a target sequence of 603 bp. (Nuraini et al., 2012).

The amplification results were qualitative analyzed using 1.5% agarose gel electrophoresis (agarose, TBE buffer, EtBr, loading dye, and 1 kB DNA ladders). The electrophoresis was performed using Horizontal Electrophoresis (Mupid 2 Plus) with a power of 50 V for 45 - 55 minutes and visualized using Chemidoc Gel Imaging (Bio-Rad / BR-200).

Table 2: Concentration and Purity of Fresh Meat DNA Isolate.

Sample	Purity (λ 260/280)	Concentration (ng/µl)
Rat	1.98	152.19
Mice	1.94	62.98
Chicken	1.72	90.20
Cattle	1.50	111.16
Horse	1.72	77.33
Lamb	1.96	392.78
Dog	1.84	50.31
Pig	1.96	60.30

3 RESULT AND DISCUSSION

Based on the results, DNA isolation performing modified Chloroform Isoamyl Alcohol (PCI) method on fresh meat samples from various non-halal animals produced DNA with concentrations ranging from 50.30 ng / µl to 392.78 ng / µl with a purity of 1.50 to 1.98 (Table 2). The highest DNA concentration was found in lamb meat while the lowest was in cattle. The protein contaminants with purity values of 1.50, 1.72, and 1.72 were detected in most of the pure DNA isolations, except in cattle, horse, and chicken. The purity values with less than 1.8 indicate, there is a presence of protein contamination, while the values more than 2.0 indicate that there's a presence of RNA contamination (Nzilibili et al., 2018). The presence of protein contamination in DNA isolation results implies a shortage of effectiveness in using Pro-K to denature all proteins in chicken, horse, and cattle meat. In the future study, a modified with the addition of Pro-K or phenol needs to be done in order to maximize the protein denaturation on the purification stage. The PCI method using proteinase on DNA isolation was more effective comparing the used of ammonium to remove the protein content (Minematsu et al., 2004; Haunshi et al., 2008).

The DNA isolation was performed based on comparing the PCI conventional method and commercial KIT to produce a high-quality DNA isolation. However, there are several inadequacy on PCI method which required a longer time and toxic reagents content: phenol, chloroform, SDS, etc. Besides, the use of commercial KIT is relatively expensive. Modification of the PCI method has been used to produce good quality DNA isolates without smear even though it was used only one purification stage (PCI and incubation with minimal time) (Haunshi et al., 2008).

The specificity test was used the DNA isolates from several animals and amplify used PCR technique. The amplification results that used 12S rRNA primers showed DNA bands in rat meat samples with sizes ranging from 228 bp.

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1    CAAAGGTTTG GTCCTGGCCT TATAATTAAT TGGAGGTAAG ATTACACATG CAAACATCCA
61   TAAACCGGTG TAAAATCCCT TAAACATTTG ---CCTAAAA CTTAAGGAGA GGGCATCAAG
121  CACATA---- -ATATAGCTC AAGACGCCTT GCCTAG-CCA CACCCCCACG GG-ACTCAGC
181  AGTGATAAAT ATTAAGCAAT GAACGAAAGT TTGACTAAGC TATTCC--TC TCAGGGTTGG
241  TAAATTTCGT GCCAGCCACC GCGGTCATAC GATTAACCCA AACTAATTAT T--TTCGGCG
301  TAAAACGTGC CAACT---AT AAATCTCATA ATAGAATTAA AATCCAACTT ATATGTGAAA
361  ATTCATTGT- ---TAGGACC TAAGCCCAAT AACGAAAGTA ATTCTAATCA TTTATATAAT
421  GCACGATAGC TAAGACCCAA ACTGGGATTA GATACCCC-A CTATGCTTAG CCCTAAACCT
481  TAATAA-TTA AACCTACAAA ATTATTTGCC AGAGAACTAC TAG--CTACA GCTTAAAACT
541  CAAAGGACTT GGCGGTACTT TATATCCATC TAGAGGAGCC TGTTCTATAA TCGATAAACC
601  CCGTTCTACC TTACCCCTTC TCGCTAATTC AGCCTATATA CCGCCATCTT CAGCAAACC-
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Figure 1: The site of the design of the primer attached to the 12S rRNA sequence *Rattus norvegicus*, note: yellow is forward primer and red is reverse primer.

The DNA band was appeared in rat samples which indicated the primers are specific to amplify the 12 rRNA gene sequence on rat (Figure 1). While, the amplification result used Cyt-b primers as the comparison gene was formed DNA bands with 603 bp in the rat meat sample. This result was verified that both 12s rRNA and Cyt-b primers are specific to amplify rat DNA sequences (Figure 2). Cyt-b primers are often used to compare the phylogenetics of species in the same genus or the same family. Several studies of the genetic diversity of the Cyt-b gene have been studied in cattle (*Bos taurus*), lamb (*Ovis aries*), and goats (*Capra hircus*), roebuck (*Capreolus capreolus*), and red deer (*Cervus elaphus*) (Wolf et al., 1999; Nuraini et al., 2012). Rats (*Rattus norvegicus*) have a long Cyt-b sequence of 1143 bp (Nuraini et al., 2012).

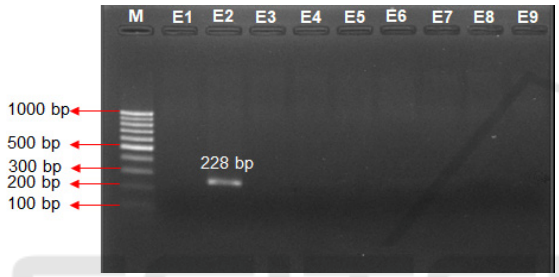

Figure 2: Result of 12S rRNA Primer. Note: E1: (-), E2: Rat, E3: Mice, E4: Chichken, E5: Cattle, E6: Horses, E7: Lamb, E8: Dog, E9: Pig, M: DNA Marker 100 bp.

The success amplifying uses PCR technique was demonstrated by the 12S rRNA and Cyt-b primers tested on various animal meat isolates producing target DNA fragments with different lengths according to the length of the targeted DNA. Cyt-b primer has been used previously to detect rat DNA fragments resulting in 603 bp of target DNA (Nuraini et al., 2012) which conforms to the results obtained in this study.

The result of the 12S rRNA primer specificity test showed that the amplified band was formed in the meat sample of rats with a length of 228 bp. It shows that the mitochondrial DNA of the 12S rRNA gene can be used as a marker or primer for specific species to identify species as it has a large variation among the species (Springer and Douzery, 1996), and 12S rRNA is mostly used in intra and inter-species phylogenetic studies (Tougard et al., 2001). In the other hand, the examined of specificity of Cyt-b primer were represent the quite clear bands with 603 bp of rat meat sample. The Cyt-B primer used in this study had quite specific properties (Nuraini, 2012). The Cyt-b primer was further examined on various samples contained some mixtures beef and pork or pork and rats with varying concentrations (1%, 5%, 10%, 15%, 20%, and 25%). As a result, the specificity of the Cyt-b primer was shown by a specific primer detecting samples of rat DNA fragments contained in a mixture of beef and rat. Meatballs which are composed of 15% rat meat would be more clearly detected compared to 1% that could not be detected due to the very small value (Nuraini et al., 2012). The succession of primers to amplify the DNA target was supported by the primers sequence contain of nucleotide bases that might specifically hybridized to DNA template (Yuwono, 2006) and a nucleotides sequence originating from the DNA target. A good primer was consisted of nucleotide bases that conserved on the template, thus not exist in any other location on its template (Pelt-verkuil et al., 2008). The succession of specifically designed of 12S rRNA primers are need further analyzed to detect the sensitivity on rat DNA fragments (*Rattus norvegicus*). In addition, the 12S rRNA primer needs to be applied in detecting rat DNA fragments in various processed food products. Thus, conventional PCR techniques using species-specific primers are qualifiable to detect DNA fragments of non-halal animals (*Rattus norvegicus*) with specifically, rapidly, and conveniently.

4 CONCLUSION

Based on the results in this study, the 12S rRNA primer specificity test on rat meat samples produced a sequence with 228 bp. Therefore, both 12S rRNA and Cyt-b are specific primers which able detect rat DNA fragments (*Rattus norvegicus*). Nevertheless, there is still need to undertake further examine on specific 12S rRNA regarding to the sensitivity of the primers in detecting rat DNA fragments to obtain the LOD (limit of detection) sample concentration and its application in various meat products.

Figure 3: Result of Cyt-b primer amplification. Note: G1: (-), G2: Rat, G3: Mice, G4: Chicken, G5: Cattle, G6: Horses, G7: Lamb, G8: Dog, G9: Pig, M: DNA Marker 100 bp.

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