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RESEARCH ARTICLE

The use of Displacement Loop mtDNA in Halal Forensic Investigation in Indonesia

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ABSTRACT:

Background: Indonesia, home to the largest Muslim population in the world, is a major potential market for halal products, yet one which is subject to frequent adulteration of imported food items. The application of halal forensic scientific methods to detect adulteration is essential in determining the halal quality of food content. **Purpose:** The purpose of this study was to evaluate the DNA content of molecular-based food products presumed to having been mixed in order to determine their halal quality. **Materials and Methods:** The research was descriptional in character using the D-Loop region for the primary design relating to six species, namely Gallus gallus (chicken), Sus sacrofa (pork), Boss Taurus (bovine), Capra hircus (goat), Equus caballus (horse), Rattus novergicus (rat) and the optimization of the cytochrom b primer as a comparative control of primary D-Loop success. **Result:** The study showed that the D-loop region can potentially be employed for investigative purposes within the halal forensic field as the primary cytochrom b mitochondrial DNA used for comparison. **Conclusion:** Primer that designed from displacement loop region and cytochrome b in mitochondria from six species can be used for determination in halal forensic as sensitive DNA amplification using PCR.

KEYWORDS: Halal Food, Molecular Detection, design primer, D-loop mtDNA, Polymerase Chain Reaction.

INTRODUCTION:

The very high global demand for halal food, with almost US$700 billion being spent annually on such products, renders products labelled as halal particularly vulnerable to counterfeiting. Moreover, the higher price of halal food compared to that of non-halal varities results in food adulteration in many countries[1-6]. The consumption of horse meat in Europe, rat meat in China[1] and, most shockingly, human flesh at McDonalds in the US[7] highlight the importance of authenticating meat species in order to give consumers confidence in brands labeled "100% halal"[1,2].

Indonesia, with the largest Muslim population and potential halal product market in the world, is susceptible to the adulteration of imported food items. To address this problem, a scientific method is required to address concerns on the part of Muslims that their food is halal. One potential method is that of applying a molecular-based detection technique, namely; Polymerase Chain Reaction (PCR) which can help to determine the DNA content of food material whose halal status is open to question. This is considering that DNA has thermal stability and was found in almost all cells of living species[1-7].

DNA also has the dual advantages of sensitivity and accuracy since it is a stable molecule which contains more information than protein[8]. This DNA-based detection technique is proven to be more sensitive and specific with the result that it can be used to detect the content of food products that have been subject to further processing[9].

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The DNA regions that can be subjected to Halal Forensic analysis are the cytochrom b segment and the displacement loop region of the DNA Mitochondrial strand. These can also be examined by means of 12S rRNA gene amplification technique or analysis of the genomic region using specific repetitive interspersed elements. According to Alraidh[10], the use of this genomic region represents an option with regard to molecular detection, although limitations exist, especially when the inspection material is boiled at temperatures above 134 degrees Celsius.

D-Loop or Displacement Loop is an area of mtDNA that does not encode polypeptides, has a high mutation rate and plays a role in the regulation of mtDNA genetic expression. This area has enormous significance for forensic examination because the sequences found in this D-loop tend to differ in various species (polimorphism). The use of D-loop in detecting the halal content of food has been largely successful, thereby promoting the use of various PCR-based methods in detecting halalness[9,10]. This study aimed to evaluate the DNA content of food materials suspected of having been mixed in order to determine the halalness of molecular-based food products.

MATERIAL AND METHODS:

This investigation formed part of the research activities of the Halal Product Research and Inspection Center of Universitas Airlangga and received ethical clearance approval from the Health Research Ethical Clearence Commision, Faculty of Dental Medicine, Universitas Airlangga Surabaya, East Java, Indonesia (No. 313/HRECC.FODM/VI/2018). The research was descriptive in character and began with the identification of DNA sequences in the D-loop region using the gene bank data of six species considered either halal or haram for human consumption. These comprised: Rattus norvegicus (rat), Gallus gallus (chicken), Capra hircus (goat), Sus scrofa (pig), Equus caballus (horse) and Bos taurus (cow). The data was used as the basis for primary design or producing synthetic oligonucleotides. Each synthetic primer or oligonucleotide was designed using the Primary 3 program implemented through the Primary-BLAST server[11,12]. This facilitated a primary search with several related targets contained in the database, such as non-redundant (nr) databases, that was, by using DNA sequences as queries. The query sequence for each species consisted of a complete sequence of coding of mitochondrial DNA in the D-Loops region. A pair of primers was then selected from several alternative outputs generated during the search. Similar protocols were used to design primary pairs for each other species. The primary D-Loop design can be seen in Table 1.

Table 1. The primary D Loop design

Species	Gen Bank accession no. of query sequence	Primer sequence	Expected amplicon (bp)
Gallus gallus Primer Cyt B Cycle	AB829473	F- GG1 5' -TGATCGCGGCATCTTCTCTC R- GG1 5' -AGGGACACGAGAGGACTAGG	155
Capra hircus	AY853278	F- 5' -CCGTCCACTAGATCACGAGC R- 5' -CTCAGACGGCCATAGCTGAG	260
Sus scrofa	AF276921	F- 5' -ACACCCTATAACGCCTTGCC R- 5' -TAGGTGCCTGCTTTCGTAGC	149
Equus caballus	AF354429	F- 5' -TTCTTCAGGGCCATTCCCAC R- 5' -GCACAGGTGTGCTTGTTTCC	261
Bos taurus	AF034439	F5' -TAACACGCCCATACACAGACC R- 5' -GCGGCATGGTAATTAAGCTCG	260
Rattus norvegicus	HQ655891	F- 5' –GAAATCAACAACCCGCCCAC R- 5' -ACGGCTATGTTGAGGAAGGC	250

11 synthetic primers or oligonucleotides used for comparison to the success of D-Loop primary design and cytochrome B primers derived from related journal were used, in the following Table 2[13].

Table 2. Sequence of cytochrome B oligonucleotides

Primer Name	Primer sequence	Product size
Bovis	F: 5' – GCCATATACTCTCCTTGGTGACA-3'	271 bp
Bovis	R: 5' – GTAGGCTTGGGAATAGTACGA-3'	
Sheep	F: 5' – ATGCTGTGGCTATTGTC-3'	274 bp
Sheep	R: 5' – CCTAGGCATTTGCTTAATTTTA-3'	
Pork	F: 5' – ATGAAACATTGGAGTAGTCCTACTATTTTACC-3'	149
Pork	R: 5' – CTACGAGGTCTGTTCCGATATAAGG-3'	
Chiken	F: 5' – GGGACACCCTCCCCCTTAATGACA-3'	266
Chiken	R: 5' – GGAGGGCTGGAAGAAGGAGTG-3'	
Donkey &	F: 5' – TTCTGCTCTGGGTGTGCTACTT-3'	221
Horse	R: 5' – CTACTTCAGCCAGATCAGGC-3'	

Sample Collection and DNA Extraction:

Samples of Gallus gallus (chicken), Capra hircus (goat), Sus scrofa (pig), Equus caballus (horse), and Bos taurus (cow) were obtained from slaughterhouses, while those of Rattus norvegicus (mice) were sourced from within the local population. Blood specimens were taken from the six species and inserted into the EDTA tube. DNA extraction using organic methods was then performed on the blood drawn by means of Trizol. 0.5ml of blood was introduced into the centrifuge tube, 1ml of DNAzol being added and subsequently vortexed. The sample was incubated for 15 minutes, with 0.2ml chloroform being added and vortexed. After incubation, the sample was centrifuged at 8,000 rpm for 15 minutes, the supernatant carefully removed and then inserted into a separate eppendorf tube. The supernatant was added to 0.5ml of isopropanol, homogenized by mixing and incubated for 15 minutes after which the sample was centrifuged at 12,000rpm for 10 minutes, the pellets then being collected and washed with 0.5ml of 70% ethanol. Those pellets containing DNA were then added to 50µl of sterile aquadest. The DNA obtained was duplicated by PCR, with DNA quantification performed by means of a nanodrop DNA quantification tool. The DNA quantification results formed the basis for the DNA doubling process using primers previously designed using the Cycler Biorad T100 Thermal PCR method.

DNA Electroforesis Method:

DNA visualization was conducted by means of 2% agarose gel, after previous immersion in 2% Ethidium Bromide solution for 30 minutes. Observation of the bands formed involved the use of a UV transilluminator with a Bio-rad gel documentation system.

RESULTS:

The results of DNA quantification obtained by means of UV nanodrop spectrophotometry can be seen in Figure 1. They show that samples can be used in the PCR optimization process with a comparable cytochrome primer b designed by Doosti et al and a D-loop primer from the study design, as well as for Species DNA sensitivity testing. The PCR optimization results obtained using a Thermal Cycler T100 and Bio-rad on cytochrome primers b designed by Doosti et al. can be seen in Table 3. The PCR product derived from optimization with the PCR program above produced a visualization of agarose gel 2% as shown in Figure 2. Cytochrome b or primary synthetic oligonucleotide primers designed by Doosti et al[13] can be used for subsequent processes, namely, as a comparative primer to determine the success of the primary design in the D-loop region.

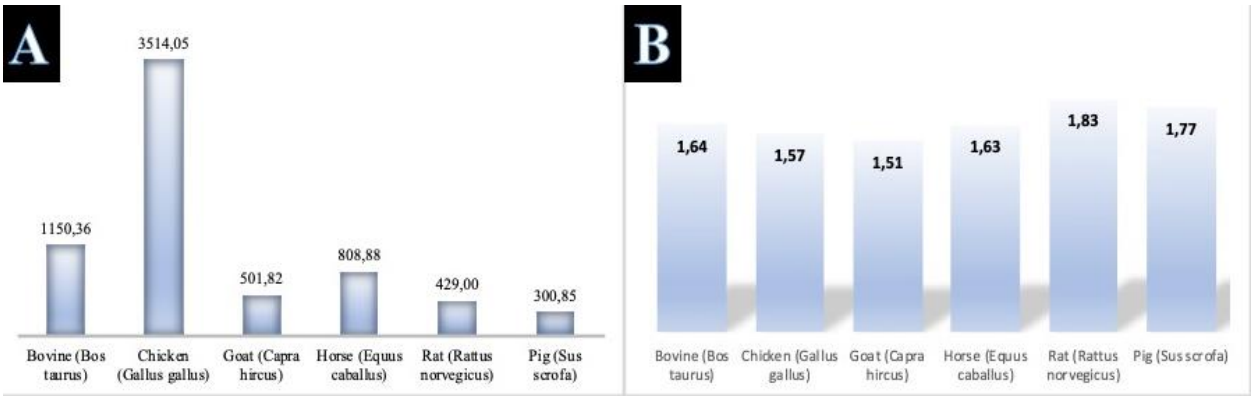

Figure 1. Quantification result of 6 species DNA 6 as control. A is the concentration (ng/ml) and the B is the purify of protein.

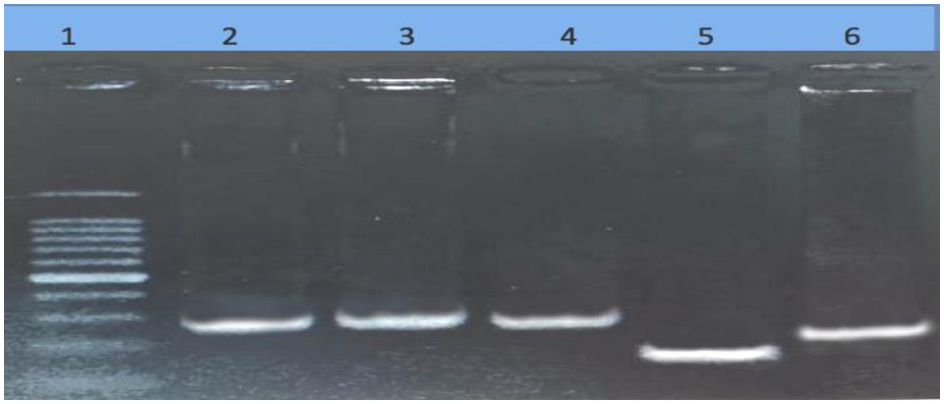

Figure 2. Amplification results from cytochrome b region. Information: Lane 1 Marker ladder 100 bp, lane 2-lane 6 with each amplicon product from DNA region cytochrome b chicken in 266 bp, goat in 274 bp, bovine in 271 bp, pig in 149 bp, horse in 221 bp

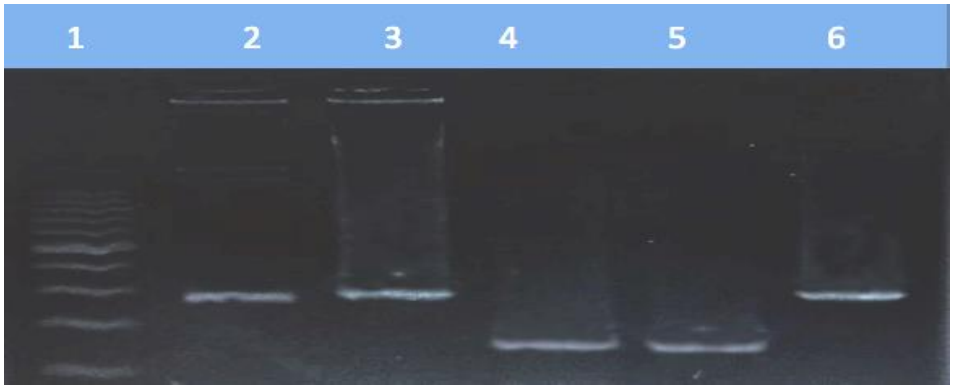

Figure 3. Amplification results from D loop region. Information: Lane 1 Ladder markers 100 bp, lane 2-lane 6 with each amplicon product from DNA region D-loop bovine in 260 bp, sheep in 260 bp, chicken in 155 bp, pig in 149 bp, horse in 261 bp.

Primary D-Loop optimization:

The PCR results of the primary design process using the program are shown in Table 4. The PCR program table was optimized using the Thermal Cycler T100 (Biorad, US) on cytochrome primers b designed by Doosti et al[12]. The multiplication of DNA by PCR using the optimization program above produces in figure 3. The electrophoresis gel results above show that the synthetic oligonucleotides from the D-loop region successfully amplify DNA from the D-loop region with an amplicon product.

Sensitivity test:

To analyze the reliability of the D-Loop primers designed, a sensitivity test with 1x to 5000x dilution was conducted. Sensitivity tests were carried out on primary design D-loop for five species successfully used in the DNA amplification process with previous PCR, plus 1 species (Rattus novergicus) as an additional species in this study. The DNA of six species diluted to 5000x could still be detected using the D-loop primer designed in this study. This means that the primary or synthetic research oligonucleotide has sensitive detection capabilities. These results illustrate that the primary design and D-Loop of this study can be used as sensitive detectors in conditions of minimal DNA concentration (Figure 4).

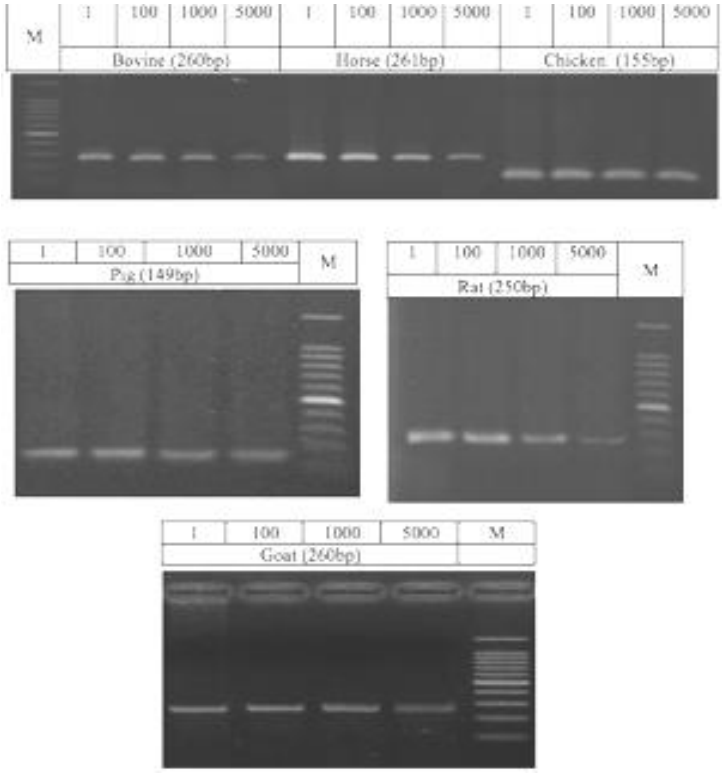

Figure 4. Electrophoresis results of the six species samples using 1x-5000x dilution.

DISCUSSION:

Many forms of halal forensic detection currently use mitochondrial DNA as an identification material due to the relatively high number of mitochondria per cell (somewhere between hundreds and a thousand). In addition, the variability of mitochondrial DNA caused by an elevated mutation rate is high compared to nuclear DNA although, according to Alraidh et al., this genomic region remains an option for use in molecular detection. There is, however, a limitation on DNA, especially when the examination material is boiled at temperatures above 134 degrees Celsius. This results in mitochondrial DNA being a superior option when compared to core DNA[14]. DNA concentrations originating from the blood of six species (mean 1117.49ng / ul and purity of 1.66), were adequate for use as DNA examination specimens in this study[15]. This means that the sample could be used in the PCR optimization process with a comparative cytochromic primer and D-loop primer from the study design, as well as for DNA sensitivity testing[13]. According to Chaobo et al[16], the stability of mitochondrial DNA (mtDNA), which is distributed throughout all tissues, is higher than genomic DNA, leading to the use of mtDNA as a gene target for species identification in many methods. There are several mitochondrial DNA regions that are used for the benefit of halal forensic detection. Mutalib et al[3] mention the cytochrom b segment and the D-loop region as the main genes of Mitochondrial DNA used in the halal forensic detection process.

In this study, the electrophoresis proved that the D-loop region on mtDNA has the ability to detect four species as well as the ability possessed by cytochrome b gene. The specifically for the other two species, it successfully detected by primers designed using DNA templates on DNA extracted from species that same. With regard to the sensitivity of the amplification products produced, in this study the template DNA amplified by PCR using D-loop primary design can be amplified to dilution of 5000x. This shows that the resulting D-loop primer has the potential to be used in samples with little DNA concentration, especially in the detection of processed food products that are grilled at temperatures above 100 degrees Celsius.

CONCLUSION:

Primer derived from the D-loop region and cytochrome b in mitochondria from six species can be used for sensitive DNA amplification using PCR that offers benefits for halal forensic detection. Further study is required to explore D-loop region potential in relation to halal forensic scientific methods.

ETHICS AND CONSENT:

All applicable institutional guidelines for the care and use of animals were followed. This research received ethical clearance approval from the Health Research Ethical Clearence Commision, Faculty of Dental Medicine, Universitas Airlangga Surabaya, East Java, Indonesia (No. 313/HRECC.FODM/VI/2018).

CONFILCT OF INTERESTS:

The authors declare that they have no competing interests

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REFERENCES:

1. Ali M.E. U. Mustafa Hashim, S, Che Man Y.B, Dhahi Th.S, Kashif M, Kamal Uddin Md, Abd Hamid S.B. Analysis of pork adulteration in commercial meatballs targeting porcine-speci fic mitochondrial cytochrome b gene by TaqMan probe real-time polymerase chain reaction. Meat Science 2012; 91: 454– 459
2. Rahman Mahfujur Md, Ali Eaqub Md, Abd Hamid Sharifah Bee, Bhassu Subha &Mustafa Shuhaimi, Al Amin Md., Abdur Razzak Md. 2. Lab-on-a-Chip PCR-RFLP Assay for the Detection of Canine DNA in Burger Formulations. Food Anal. Methods 2015; 8:1598–1606.
3. Mutalib Sahilah Abd., Wan Nazri Wan Sakeenah, Shahimi Safiyyah, Yaakob Norhayati, Abdullah Sani Norrakiah, Abdullah Aminah, Babji Abdul Salam and Ghani Maaruf Abd,. Comparison Between Pork And Wild Boar Meat (Sus Scrofa) By Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (Pcr-Rflp). Sains Malaysiana 2012; 41(2): 199–204
4. Jeanette, T. ,2013. "Chinese police seize more than 20,000 kg of fake beef". Available via http://my.news.yahoo. com/blogs/what-is-buzzing/chinese-police-seize-more-20-000kg- fake-beef-035448883.html Accessed 14 Sep 2018
5. Ulca P, Balta H, Cagin I, Senyuva HZ. Meat species identification and Halal authentication using PCR analysis of raw and cooked traditional Turkish foods. Meat Sci; 2013 94(3): 280–284.
6. Hsieh YHP, Woodward BB, Ho SH. Detection of species substitutioln in raw and cooked meats using immunoassays" J Food Prot 1995; 58(5):555–559
7. Alikord Mahsa, Momtaz Hassan, Keramat Javad, Kadivar Mahdi, Rad Homayouni Aziz. Species identification and animal authentication in meat products: a review. Food Measure 2017; 30: 89-93
8. Lim, A. C. And Wong, C.M.V.L, 2013. "Development of Techniques Based On Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) For Meat Identification (Halal & Non-Halal)" University Malaysia Sabah (Ums) Funding Under The Fundamental Research Grant (Research Grant No.: B-0901-13-Pr/U023).
9. Asma Al-Tamimi, Yaquob Ashhab, 2012. "Identification of Meat Species And Meat Products By A Multiplex PCR Method". Biotechnology Research Center, Palestine Polytechnic University, Hebron, Palestine
10. Alaraidh Ibrahim Abdullah, 2008. "Improved DNA Extraction Method For Porcine Contaminants, Detection In Imported Meat To The Saudi Market". Saudi Journal Of Biological Sciences;15 (2): 225-229
11. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M and Rozen SG. Primer 3--new capabilities and interfaces. Nucleic Acids Res 2012; 40(15): e115
12. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden T. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 2012; 13:134
13. Doosti A, Ghasemi PD, Rahimi A, Molecular Assay to fraud identification of meat products" J Food Science Technology 2014; 51(1):148-152
14. Norrakiah, A.S., Shahrul Azim, M.G., Sahilah, A.M. and Abdul Salam, B. Halal analysis of raw materials, ingredients and finished bakery products using PCR and gene chip southern-hybridization for detection of porcine DNA. International Food Research Journal 2015; 22(5): 1883-1887
15. Sosiawan Agung, Raharjo Dadik, Nuraini Indah, Kartikasari Nadia, Nugraha Patera Alexander and Dimas Muhammad Aditya Ari. Detection of short tandem repeats at 5 loci and amelogenin with cell-free fetal DNA as a specimen in the development of prenatal paternity diagnostic tests" Egyptian Journal of Forensic Sciences 2018; 8: 15.
16. Chaobo, X., Feng, G.., Pingya, W., Jin, Z. and Yuanyuan, L. Development and utility of a tri-primer PCR for simultaneous detection of cattle, sheep and pig in meat products. International Food Research Journal 2017; 24(1): 159-163.