Methods for Detection of Foods, Cosmetics, and Drugs Through A Mitochondrial DNA Analysis (An Overview of the Molecular and the Qur'anic Aspects)

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Abstract. The halalness and originality of foods, cosmetics, and drugs are not only a national issue but also a global one. The community demands for halal foods, cosmetics and drugs are related to the consumer rights to obtain products that are comfortable and safe. The legality of a product is determined from upstream to downstream so that it is produced and ready to use or consume. One method for the detection of product halal analysis is Polymerase Chain Reaction based on mitochondrial Deoxyribonucleic Acid (mt-DNA). Mt-DNA is often used for the analysis of species evolution or species identification because it has a high mutation rate and maternal inheritance in which recombination does not occur so that the lineage can be directly determined. Part of mitochondrial DNA that is used for a specific marker is Cytochrome b. This paper is a review of halal foods, cosmetics, and drugs in Islamic and molecular aspects using several methods such as Polymerase Chain Reaction (PCR), PCR-Restricted Fragment Lenght Polymorphisms, Multiplex PCR, and Real-Time PCR. It also explained several factors that influence the level of accuracy of halal products through these methods.

Keywords: Mitochondrial DNA, Detection, Halal, PCR, Product.

1 Introduction

Guaranteed halal products such as foods, cosmetics, and drugs is a necessity for Muslims nationally and internationally. According to the Halal Product Guarantee Act (UUJPH) of Indonesia No. 33 of 2014 [1], halal product guarantee does not only cover foods, cosmetics, and drugs but have a large scope, including chemical products, biological products, genetic engineering products, as well as used goods. The legality of a product is determined from upstream to downstream so that the product is produced and ready to used or consume, later known as halal product processing.

The halal product process is defined as a series of activities to ensure the halalness of the products, including the supply of materials, processing, storage, packaging, distribution, sales, and product presentation [2]. The purpose of guaranteeing halal products is to provide comfort, security, safety, and certainty for the availability of halal products for the community and increase added value for the producers.

The truth of a halal statement on the product label is not only proven in terms of raw materials, food additives, or auxiliary materials used in producing products but must also be proven in the production process. Every person who manufactures or imports product

This paper was presented on 19th Annual International Conference on Islamic Studies (AICIS), October 1st- 4th in Mercure Batavia Hotel, Jakarta

AICIS 2019, October 01-04, Jakarta, Indonesia

DOI 10.4108/eai.1-10-2019.2291746

packaging into Indonesian territory for trading must be checked by an inspecting institution that has been accredited in accordance with the provisions of the legislation in force. The implementation of UUJPH began this year because it has been five years since the UUJPH law has been enacted. However, the implementation in the field has not been yet optimal [2].

There are many methods to detect halal products, such as protein analysis, Gas Chromatography (GC) method, electronic nose, a spectroscopic method using infrared, nuclear magnetic resonance spectroscopy, differential scanning calorimetric and Polymerase Chain Reaction based on Deoxyribonucleic Acid (DNA).DNA is unique and specific to each organism. The uniqueness and specificity of DNA could be found between species, intra-species, and between individuals within the same species. The use of DNA to detect halal products is more accurate than other methods as the reported detection of pork contamination and mixture of pig DNA and rat DNA at ≥ 1% level in meatballs beef detected properly using the CytB gene as a marker [3], a mixture of ≥ 5% pork in boiled beef [4], soft gel candy derived from pig gelatin [5], 1 pg pig DNA in boiled processed beef [6], 0.01 ng pig DNA in lipstick [7]. In addition, mitochondrial DNA markers are more sensitive, accurate, relatively inexpensive, and could be used in processed products that have been mixed, boiled, physical treatments, and in degraded DNA. The non-halal ingredients in cosmetic products and drugs such as the use of pig and placenta, or the use of fanged animals such as tigers in traditional Chinese medicines [8] or pigs and their derivatives such as lard, oil, and gelatin which used in lipstick components [7] can also be detected using the PCR method. The methods with high accuracy include PCR, PCR-RFLP, Duplex and /or Multiplex PCR and Real-Time PCR.

2 Method

2.1. Halal Foods, Cosmetics and Drugs in Islamic and Consumer Perspective

Something that is not allowed in Islam is called haram. Halal is the opposite of haram, as explained in the Qur'an Surah Al-Araf (7):157; Al-Mukminun (23):51; Al-Baqarah (2):172; Almaidah (5):88. A Hadith narrated by Muslim explained that"The halal is clear and the haram is clear; and between the two there are things that are *musyabihat* (doubtful, vague, unclear halal haram) (H.R. Muslim). The halalness of a product covers the substance and the way to get it. Halal is not only limited to foods and drinks, but it also includes cosmetics, drugs and other things being used [9]. Basically, all materials are halal. However, there are texts that declare their lawfulness according to Islamic law, i.e. the Qur'an, Hadith, Ijma Ulama, and Qiyas.

Diversification of food products, cosmetics, and medicines as well as the development of processing technologies, complex processing processes, various materials such as raw materials, additives, stabilizers, solvents, etc., are used in the processing, which of course affects the halalness of the products. Thus, advanced biotechnology in the processing of foods, cosmetics, and medicines, can increase the values and the benefits of the products. Unfortunately, not all products produced from biotechnology are guaranteed to be halal for several reasons, i.e. originating from haram materials, the use of non-halal additives, contaminated with substances that are unclean materials in processing, packaging, and distribution.

A plant-based source in its origin is halal except the materials are intoxicated or health-threatening. Animal origin ingredients that are declared haram can be grouped into 9 groups: 1) Carrion; 2) Blood; 3) Pigs and their derivatives such as pork, lard, and gelatin; 4) Halal animals but slaughtered not for God; 5) Animals died of being suffocated; 6) All drinks that

are choked intoxicants, including khamar and alcohol; 7) fanged animals, such as lions, dogs, wolves, and tigers; 8) birds with sharp claws; 9) reptiles like frogs and snakes. Material derived from microbes and materials produced through chemical processes, biological processes and genetic engineering processes are forbidden if the growth and manufacturing process are mixed, containing or being contaminated with the prohibited materials [9].

The critical point in food processing can occur in the manufacturing process or in ingredients added. The utilization of biotechnological advances in food processing has been beneficial in improving the physical and chemical quality of food products produced, such as the use of enzymes for the preparation and processing of materials, microbial technology to produce fermented food or food additives, and plants or animals genetically modified.

There are several critical points for haram cosmetics [3], namely 1) Ingredients used, such as pigs and derivates (QS Al-Baqarah: 173), placenta or parts of human organs (Fatwa MUI Number: 2 / MunasVI / MUI / 2000), placenta-derived from halal animal carrion (MUI Fatwa Number: 30 of 2011); 2) Sources of active substances such as collagen and elastin. Collagen is usually extracted from animal bones. The animals used must be halal and the halal animals should be slaughtered in a halal way, and are not decaying; (3) Sources of stabilizers ingredients such as lauryl palmitate, glycerol monostearate; (4) Sources of essential fatty acids, such as linoleic, linolenic and arachidonic which function as anti-oxidants and stabilizers for these fatty acids to prevent oxidation; (5) Hormones and glandular extracts such as placental protein extracts, placental enzymes and placental lipids [4]. The four components of substances in medicine related to halalness: 1) Insulin, because pig insulin is similar to human insulin, so the use of pig insulin is cheaper than human insulin in processing; 2) Heparin is an anti-coagulation agent of blood vessels, heparin can be produced by pigs; 3) Gelatin is a protein derived from animal collagen, animals that are often used are pigs; 4) Alcohol [5]. The use of pigs as animals producing substances needed to produce drugs, food, and cosmetics, considering that these pigs have several advantages, namely1) prolific (producing many piglets per birth); 2)converting rations into meat efficiently; 3) shorting harvest life;4) high percentage of carcasses [6], 6-8 month calving interval, and 6-12 litter size [7].

The critical points of halal drugs can be known in 1) active ingredients used, excipients and auxiliaries used; 2) ensure production facilities used specifically for halal products only; 3) ensure there is no chance of being mixed and contaminated with illegal substances; 4) halal packaging; 5) material and equipment purification and purification [14]. For this reason, it is very important to do labeling and halal certification for cosmetics so that it can provide a sense of security and comfort for the users. However, cosmetics consumers are so far a lack of care about the halal label [15].

2.2. DNA Review in Science and The Qur'an

DNA (Deoxyribonuclease acid) is a unit of inheritance of the nature of living things from one generation to the next generation. DNA consists of a series of nucleotide bases consisting of Adenine (A), Thymine (T), Cytosine (C) and Guanina (G) which are strongly phosphodiester bonds. Humans and animals have 2 kinds of DNA, namely nuclear DNA and mitochondrial DNA, in contrast to plants that have nuclear DNA and chloroplast DNA. Nuclear DNA is always paired, where a sequence is inherited from the mother and another sequence inherited from the father. The strands of nuclear DNA formed double helix-like twisted stairs. DNA is the blueprint of living organisms. This is written in the Qur'an Al Infithaar (82):8. This verse states that there is the potential possessed by every living creature

to be able to express the performance that can be observed visually that is good or bad. That potential is known as genetic or more specifically, it is called DNA.

In The Qur'an, Surah Abasa (42):19, Allah has explained that humans were created from spermatozoa and then "determined" it. A spermatozoon could be interpreted as a male gamete heritable from father chromosomes. The millions of spermatozoa are produced by male humans or animals, however, there is only one spermatozoon that penetrates the cumulus oophorus (the layer that protects the ovum) ovum which carries another pair of DNA sequences. After the fertilization process, the two sequences were formed a double-strand known as the double helix. Once the glorious creation of living things by Allah Swt. who has created living creatures on this earth in the best possible creation? This shows the greatness of its creator, namely Allah Swt. as explained in the Qur'anSurah At-tin (95):4

Mitochondrial DNA (mt-DNA) is only inherited from the mother, circular shape, found in the mitochondria cells, consists of 13 genes, 22 transfer RNAs, 2 ribosomal RNAs (12 S and 16SrRNA) and one non-coding region called the D-Loop region. The 13 protein genes that play a role in oxidative phosphorylase consist of 7 complexes I subunits (ND1, ND2, ND3, ND4, ND5, and ND6) and one complex unit 3 (Cytochrome B), 3 complex IV subunits (COX I, II and III) and 2 complex subunits 5. These genes have unique characteristics to specific species so that they can be used as DNA barcodes for certain species. The mitochondrial DNA has some uniques,i.e. no intron, smaller, no recombination, that could be used as a genetic marker between species, intra-species, the rate of evolution and inter-species genetic distance, and phylogeny [8]. The parts of mitochondrial DNA that are commonly used as species markers are the Cytochrome B (Cyt B) gene and the Cytochrome Oxidase I (COI) gene. The advantages of the COI gene for species markers are 1) the relatively short gene at around 648 bp 2) it is not easily mutated (relatively stable) 3) the level of variability is relatively low in species around 1-2% 4) easily amplified so that the COI gene is also used as a DNA barcode for several species [9].

3 Results and Discussion

3.1. PCR and Primers Specific Species

Polymerase chain reaction (PCR) is a technique of propagation of target DNA fragment in vitro consisted of three stages: denaturation, annealing, and extension used polymerase enzymes and deoxynucleotide triphosphates (dNTPs) with time and temperature regulation. The target DNA fragment is determined based on a specially designed primer. The several forward primers and reverse primers sequences used in detecting halal products were presented in Table 1.1.

The identification of halal products based on mt-DNA is believed to be more accurate because it used specific, reproducible, sensitive primers, rapid processing time and low cost[10], [11]. Mt-DNA is more stable than nucleus DNA and is resistant to high temperatures during the cooking process, the pressure and mixing of other ingredients and low concentration DNA of about 0.01 ng can be used to PCR amplification [11].

The PCR process takes place on a thermocycler machine automatically in accordance with the time and temperature settings. Stages on the PCR machine consist of pradenaturation, denaturation, annealing, and extension, where the PCR process is repeated 25-35 times [12] and continued with the final extension and cooling process. The crucial point in this PCR technique is to determine the temperature and time of annealing. Annealing is the primer attachment to the target DNA fragment to be multiplied. Annealing occurs at a temperature of

36^0C-72^0C [12] after DNA denaturation (breaking of hydroxy bonds (OH) from double-strand to single strand) at a temperature range of 94^0C -95^0C for 15-30 seconds.

The right temperature and time of the denaturation process will have an impact on the success of the primer attachment to the target DNA. Denaturation time that is too fast or an inappropriate temperature can cause renaturation to take place quickly so that the PCR process does not work as well as denaturation time that is too long to have an effect on the enzyme Taq polymerase enzyme is not optimal. The annealing temperature and time are crucial points in the success of PCR.

Table 1. Forward dan Reverse PrimerSequences for Detection of Halal Products

NO	Primer (5'-3')	Sequences	Annealing (^{0}C)	Amplicon Lenght (bp)	Species	Refer.
1.	Forward	5'-GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA AA-3'				[13]
	Reverse	5'- CTC GAC AAA TGT GAG TTA CAG AGG GA-3'		157	Goat	
	Reverse	5'-AAG ATA CAG ATG AAG AAG AAT GAG GCC-3'		227	Poultry	
	Reverse	5'-CTA GAA AAG TGT AAG ACC CGT AAT ATA AG-3'		274	Cattle	
	Reverse	5'-CTA TGA ATG CTG TGG CTA TTG TCG CA-3'		331	Sheep	
	Reverse	5'- GCT GAT AGT AGA TTT GTG ATG ACC GTA-3'		398	Pig	
	Reverse	5'- CTC AGA TTC ACT CGA CGA GGG TAG TA-3'		439	Horse	
2.	Forward	5'- TCT TGC AAA TCC TAA CAG GCC TG-3'				[14]
	Reverse	5'- TTT GCA TGT AGA TAG CGA ATA AC-3'		130	Pork	
3.	Forward	5'-GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA AA-3'				[10], [15]
	Reverse	5'-AAG ATA CAG ATG AAG AAG AAT GAG GCC-3'	60	227	Chicken	
	Reverse	5'-CTA TGA ATG CTG TGG CTA TTG TCG CAA AT-3'	60	331	Sheep	
	Reverse	5'-TAG CCA TGA CCG TAA ACA ATA GC-3'	60	319	Tiger	
	Reverse	5'-TTG CTA GAG CTG CGA TGA TGA AA-3'	60	523	Dog	
	Reverse	5'- AGG GGT TGT TAG ATC CTG TTT CA-3'	60	568	Cat	
	Reverse	5'- GAA TGG GAT TTT GTC TGC GTT GGA GTT T-3'	60	603	Rat	
4.	Forward	5'-CCA TCC AAC ATC TCA GCA TGA AA-3'				[11]
	Reverse	5'- GCC CCT CAG AAT GAT ATT TGT CCT-3'	55	359	Pig	
	Forward	5'- GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA-3'				
	Reverse	5'- GCT GAT AGT AGA TTTGTG ATG ACC GTA-	55	398	Pig	

		3’				
5.	Forward	5’-ATG AAA CAT TGG AGT AGT CCT ACT ATT TAC C-3’				
	Reverse	5’-CTA CGA GGT CTG TTC CGA TAT AAG G-3’	60	149	Pig	[16]
6.	Forward	5’-CCC CGT CTC CTT CCT CCG GTG GTT GAT G-3’				
		5’-CTA GAA AAG TGT AAG ACC CGT AAT ATA AG-3’	49	274	Pig	[17]

The annealing temperature depends on the size of the primer base (18-25 bases), the GC content is 50-60% and the melting temperature for both primers should be the same. The annealing temperature of several pairs of primers specific to animal species used for the detection of halal products is presented in Table 1. The next stage of the PCR process is elongation, where primers with the help of the Taq polymerase enzyme would form DNA strands in accordance with DNA sequences that are split with the speed of preparation of 35-100 nucleotides per second. The temperature used at this stage is 72^0C for 30 seconds to several minutes depending on the length of the target’s amplicon/DNA. The success of the PCR process will be known through agarose gel electrophoresis by staining with Ethidium Bromide or PicoGreen through the appearance of a single and firm band that can be observed after exposure to Ultra Violet light on GelDoc.

3.2. PCR-RFLP

PCR RFLP is a method of identifying the diversity of genes or DNA by utilizing restriction enzymes in the target DNA. Restriction enzymes are enzymes that can cut DNA from the inside, so they are also called endonuclease enzymes. Restriction enzymes cut DNA at specific sites (recognition site) during the incubation period. Each type of restriction enzyme has a different recognition site (Table 2). The mechanism of the PCR RFLP method begins with the isolation of the DNA sample and continues with PCR using specifics primers. The PCR product was then incubated using specific enzymes (Table 2). The visualization of DNA cut by restriction enzymes could be observed under the band of UV light after electrophoresis of agarose gel.

Table 2. The Names of Restriction Enzymes, Sources Organisms and Restriction Site

Enzyme Name	Restriction Site (‘5-----‘3)	Source Organisms
EcorI	G↓AATC	*Escherechia coli*
HindIII	A↓AGCTT	*Haemophilus Influnzae*
HhaI	GCG↓C	*Haemophilus haemolyticus*
TaqI	T↓CGA	*Thermus aquaticus*
BsuRI	GG↓CC	*Bacillus subtilis*
BalI	TGG↓CCA	*Brevibacteriumalbidum*
NotI	GC↓GGCCGC	*Nocardia otidis-caviarum*
BamHI	G↓GATCC	*Bacillus amyloliquefaciens*
SmaI	CCC↓GGG	*Serratia marcescens*
AluI	AG↓CT	*Arthrobacter luteus*
MspI	C↓CGG	*Moraxella sp.*
PstI	CTGCA↓G	*Escherichia coli ED8654*
PvuI	CGAT↓CG	*Escherichia coli*
SmaI	CCC↓GGG	*Seratia marcescens*

RsaI	GT↓AC	*Acidiphilium facilis*
HinfI	GT↓AC	*Haemophilus influenza R*
HaeIII	GG↓CC	*Haemophilus aegyptius*

3.3. Multiplex and Duplex PCR

Multiplex PCR is the process of amplification of several loci in the same PCR reaction using one universal forward primer with several reverse specific primers (more than 2 reverse primers) as a characteristic of certain species. If there are only two reverse primers used in the PCR process, it is known as duplex PCR. The Multiplex and duplex PCR has been applied in many areas of DNA testing i.e gene deletion analysis, mutation and polymorphism detection, quantitative analysis, and reverse-transcription (RT)-PCR, identification of viruses, bacteria parasites[18], [19] and halal product analysis [10], [20].

3.4. Real-Time PCR

Real-Time PCR (RT PCR) is a technique used to quantify the DNA target via monitor the progress of a PCR reaction, another name is quantitative PCR (qPCR).In qPCR, the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule as a known probe or dye.

3.5. The Factors That Influence The Success of PCR-Mitochondrial DNA Method in Halal Product Analysis

The success of the PCR-Mt DNA method is known by reading the band on GelDoc after electrophoresis on agarose gels. The PCR process was decided to succeed if a single, bright and clear band appeared in accordance with the target DNA. Some of the causes of the unsuccessful PCR, RFLP, Multiplex/Duplex PCR and RT PCR processes, as well as solutions to overcome these problems, are summarized in Table 4.

Table 3. PCR, RFLP-PCR and Multiplex/Duplex PCR Failure and Solution

No	Hasil PCR	Failure	Solution
1	No Band	Non Spesific Primer	Use Alternative Primer
		No Denaturation DNA	Up denaturation temperature and or add denaturation time
		Miss Annealing Temperature	Optimation of annealing temperature
		No Matching Cycles	Add more cycles
		Low DNA template/DNA Degradation	Sample Extraction Again
		Low-Quality Materials PCR	Change new materials
		Non Specific DNA/sampel	Sample Extraction Again
		DNA Contaminant with other	Sample Extraction Again
2.	Unclear Band	High-Temperature annealing	Turn down annealing temperature
		Low cycles	Add more cycles
		Need promotor matters	Add MgCl
3.	Multiple Band	Low-temperature Annealing	Up annealing temperature
		Much more Mg^{2+} concentration, Much DNA template	Recounting and PCR repeat
		Long-time extension step and More PCR Cycles	PCR Optimation
4.	Low Value of Efficiency (E)	Lack of pipetting and low DNA concentration	Repeatability Test

	on Real-Time PCR		
5.	High Value of Efficiency (E) on RT PCR	Present Inhibitor The Mixture with High Concentration	Repeatability Test

4 Conclusion

DNA as a blueprint of living things has been stated in the Qur'an along with its uniqueness. The specificity of DNA possessed by each species can be used as a species-specific marker. The use of the mt-DNA-PCR method in the detection of halal products has proven to be accurate, reproducible and relatively non-expensive for use in food, cosmetics, and drugs. The success of this method depends on the proper sample isolation technique, the quality of the sample DNA produced, the used species-specific primers, the quality of the materials used and the correct annealing temperature.

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This paper was presented on 19th Annual International Conference on Islamic Studies (AICIS), October 1st- 4th in Mercure Batavia Hotel, Jakarta