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DETECTION OF PIG DNA FRAGMENTS IN HALAL UNLABLED LIPSTICK SAMPLES USING CONVENTIONAL PCR

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ABSTRACT

From a Muslim perspective, it is very important to know the content, raw materials, and processing of the raw materials used in the cosmetic products used. One type of cosmetics that is most often used is lipstick. However, many lipsticks circulating in Indonesia are not equipped with a halal logo. One of the ingredients for lipstick is pork derivatives. These pig derivatives can be detected using PCR. Based on this background, this study aims to test the presence of pig DNA in lipstick samples that have not been certified halal on the market using 4 combinations of pig DNA fragments coding primers by using the conventional PCR method. Five commercial lipstick samples were selected by purposive sampling. DNA isolation was carried out according to the Wizard Promega Universal Kit. The PCR process was carried out with temperature optimization as follows: Predenaturation 98^{o}C: 2 minutes, denaturation of 95^{o}C: 30 seconds, Annealing 61^{o}C: 30 seconds, Extension 72^{o}C: 40 seconds, and Postextension 72^{o}C: 3 minutes. The results showed that of the 5 samples tested by PCR using 5 kinds of primer combination, none of the samples were suspected to contain pork DNA. DNA isolation is the most difficult step in the lipstick sample detection process. Even though the detection result is negative, it is necessary to carry out further tests which become the Gold Standard of DNA-based testing using Real Time PCR.

Keywords: ***Pig DNA detection, Lipstick without halal logo, halal certified, Conventional PCR***

Introduction

Along with the times, a beautiful and pleasing physical appearance has become a basic necessity for humans, especially for women. To support this, cosmetics are a major requirement to support one's appearance. Cosmetics themselves are ingredients or preparations that are used on the external body (epidermis, hair, nails, and external genital organs) or teeth and oral mucosa that function to clean and care for the body, and change or improve a person's appearance (Anggraini & Ginting, 2017).

As a country with the largest Muslim majority in the world, the halal haram of consumer products is a sensitive issue.It is very important to know the content, raw materials, and processing processes of the raw materials used in the cosmetic products used. This is in accordance with Islamic law which requires Muslims to only be allowed to consume and use halal products (Mahdiyyah & Putriana, 2019).

The commandment to consume and use everything lawful and stay away from what is haram has been explained by Allah SWT in Q.S Al Baqarah: 173 "Indeed, Allah only forbids for you carrion, blood, pork, and animals that (when) are slaughtered (called names) other than Allah. But whoever is in a state of compulsion (to eat it) while he does not want it and does not (also) transgress,

there is no sin for him. Allah is Forgiving, Most Merciful "(Al Qur'an 2: 173).

In Indonesia, the regulations regarding the distribution of halal-certified products are described in the Republic of Indonesia Law No. 33 of 2014 Article 4 which reads "All products that enter, circulate and are traded in Indonesia must be certified halal". What is included in this context are all products in the form of food, beverages, cosmetics and other disposable products. Products that fall into the halal category have several criteria. The criteria for halal cosmetics itself include not containing human body parts, ingredients, or any animal that is prohibited for Muslims, and must be slaughtered according to Islamic procedures, not containing genetically modified organisms deemed unclean, not containing khamr (alcoholic drinks), does not contain unclean contamination during preparing, processing, manufacturing and storage, and is safe for consumers (Syamsu, 2020). In addition, it does not contain animal fats or harmful chemical compounds which are declared not to be used by Muslims (Mahdiyyah & Putriana, 2019).

One of the most commonly used cosmetics is lipstick (lip color). Lipstick is a type of cosmetic makeup (decorative) which in its use only adheres to parts of the body that are made up and is not absorbed into the skin and permanently changes deficiencies (Anggraini & Ginting, 2017). Based on the results of BPOM supervision, there have been many lipsticks on the market with a wide variety of colors, types and prices (Ulya, 2018). However, there are lipstick products that contain ingredients that cause side effects such as allergies and irritation so they are not safe to use (Umaira, 2019).

In terms of quality, lipsticks must meet the following requirements in order to produce lipsticks that are in accordance with the demands of the community: a) can cover the lips completely, b) have long lasting durability, c) adhere perfectly to the lips but do not cause a sticky feeling, d) does not cause side effects such as allergies and irritation, e) is able to moisturize the lips, f) has an attractive appearance, both in terms of color and shape, and g) smooth surface, does not pock, and does not drip oil (Umaira, 2019).

The main components in lipstick include vegetable oil which functions as a solvent and dispersing agent for dyes, waxes (carnauba wax, candelilla wax, beeswax, ozokerite, spermaceti and cetyl alcohol) which function to maintain the shape of the lipstick and keep it solid, fat to form a film layer on the lips and increases the resistance of lipsticks, and dyes (Simatupang, 2018). The coloring agents used in lipstick can be in the form of natural dyes such as dragon fruit which produces red or carrots which produce an orange color, or artificial dyes such as Rhodamin B (Umaira, 2019).

Lipstick also contains several additional ingredients such as preservatives (methyl paraben and propyl paraben), antioxidants that function to protect oils and other unsaturated materials from oxidation reactions provided that the antioxidants used are odorless, non-toxic and do not change after a long storage, and perfume (Umiara, 2020). The fat component in the lipstick composition usually uses vegetable oil or mineral oil. However, in some cases, the oil component used in the lipstick is replaced with lard (Umiara, 2020). Especially for products that do not have halal certification.

The demand to always look beautiful makes Muslim consumers in Indonesia use products carelessly without paying attention to the presence or absence of a halal logo on the products used. The majority of people prefer to be able to look beautiful at an affordable cost is one of the reasons why there are still many cosmetic products that do not have a halal certificate on the market (Rachman, 2018).

One of the detection methods that are often used to test pig components and their derivatives in a product is a DNA-based detection method, namely the PCR (Polymerase Chain Reaction) method (Adzakiyyi *et al., 2020)*. The PCR method is a DNA amplification-based method that has higher specifications and sensitivity compared to other methods (Fadhlurrahman *et al*., 2015).

PCR is a molecular technology used to reproduce specific DNA fragments in vitro with primers complementary to the target DNA, resulting in thousands to millions of DNA copies. The stages in PCR are divided into three, namely denaturation, primary attachment (annealing), and elongation (Andriyani *et al*., 2019). The method of molecular technology for detecting pork and its derivatives quickly and effectively is the PCR method, both conventional PCR, Multiplex PCR, and Real-Time PCR. (Rachmawati *et al*., 2019).

Researcher using mitochondrial DNA about detection of pig DNA ini several sample contents. These genes are most often used as markers for animal detection including cytochrome b (cyt b) gene (Adzakkiyi *et al., 2020)*. Some researchers have used cytochrome b (cyt b) gene to distinguish material from different animal species (Rokhim *et al*., 2021). Rokhim *et al* (2021) state that sequence variation in cyt b causes this gene widely used as a marker for grouping animal species. The peculiarities of cyt b gene include almost same areas

for all types of animals, but there are also regions that are specific to each type of animal (Primasari, 2011). The use of pig cyt b gene for detection has been widely used in research. Pig cyt b gene is very sensitive and accurate to detect whether a sample contains pig components or not. As research conducted by Ali *et al*. (2012), Yusop *et al*. (2012), Kumar *et al*. (2014).

Based on this background, this study aims to test the presence of pig DNA in lipstick samples that have not been certified halal on the market using pig DNA fragment primers using conventional PCR methods.

Materials and Methods

The research was conducted in January 2021 at the integrated laboratory, Genetics and Tissue Culture Lab, UIN Sunan Ampel Surabaya (UINSA). Five samples of commercial lipsticks were selected by purposive sampling with the following criteria: no halal label, affordable prices, many traded in online shops and shops circulating in Surabaya.

Materials and Tools

The materials used in the study were five kinds of lipsticks without the halal logo, the Universal DNA Isolation Kit Wizard Promega: Nuclei Lysis solution; RNAse; protein precipitation solution; DNA Rehydration Solution, isopropanol, 96% ethanol, Go Taq® Green Master mix, Forward and Reverse primers, nuclease free water, agarose gel, DNA Diamond nulcei acid dye, buffer, loading dye and tissue, benchtop 100 bp ladder.

The tools used in this study were a spectrophotometer (Biochrom Biodrop-DUO), thermocycler (Labnet MultiGene Optimax), microcentrifuge (Thermo Scientific Heraeus Fresco 21), electrophoresis (Mupid2Plus), hot plate, analytical balance, microtube, spatula, micropipette and vortex, Enduro GDS-1302 gel documentation.

DNA Extraction

A total of 0.5 grams of lipstick samples were homogenized with 600 µl of Nuclei Lysis Solution and incubated at 65 ° C for 15 minutes. Samples were added with 3 µl of RNAse, then incubated again for 15 minutes at -37 ° C and cooled for 5 minutes at room temperature. The sample was added 200 µl of Protein Precipitation Solution, then vortexed and cooled in the freezer for 5 minutes. The cooled sample was then centrifuged for 4 minutes at a rate of 15,000 x g at room temperature, 600 µl of isopropanol was prepared, put the clear supernatant in the tube, and inverted. Centrifuged for 1 minute at 15,000 x g at room temperature. Then the supernatant was removed and 600 µl of 70% ethanol were added, again centrifuged for 1 minute at the same speed at room temperature. The ethanol is removed and the pellets are air-dried for 15 minutes. Then, 100 µl of the DNA rehydration solution was added by incubating at 65 ° C for 1 hour, or overnight at 4 ° C.

Concentration and Purity of DNA Isolates

Tests were carried out using a spectrophotometer (Biochrom Biodrop-DUO). 1 µl blank DNA Rehydration Solution is dropped onto the pedestal. Press the "BLANK" part on the screen to measure the blank, after that, clean the pedestal again with a tissue. Then a solution of 1 µl of DNA isolate was taken using a micropipette and put into a pedestal. Click the 'Measure' button so the screen will show the spectrum and the amount of concentration calculated.

Amplification of Specific DNA Fragments

Amplification was carried out using the PCR method, with the composition of: 2.5 µl of DNA sample, 12.5 µl of Go Taq® Green Mater mix, 1 µl of Forward and Reverse Primer each, and 9 µl of Nuclease Free Water into microtube and homogenized using vortex. Furthermore, the amplification process was carried out on a thermocycler machine (Labnet MultiGene Optimax(. The PCR process was carried out with temperature optimization as follows: 1) 98^{o}C predenaturation for 2 minutes, 2) 95^{o}C denaturation for 30 seconds, 3) 61^{o}C annealing for 30 seconds, 4) 72^{o}C extension for 40 seconds, and 5) 72^{o}C postextension for 3 minutes. After the process is complete, the tubes are taken and stored at room temperature or at 4^{o}C until further analysis. The 4 primary combinations used in the study for PCR amplification were as follows:

Table 1. Primary Amplification of Pig DNA Coding Target Genes

No.	Target Gene	Primer	Sekuens 5'3'
1	Cyt b	Pork F	ATG AAA CAT TGG AGT AGT CCT ACT ATT TAC C
		Pork R	CTA CGG GTC TGT TCC GTT GG
2	Cyt b	Pork R	AAC CCT ATG TAC GTC GTG CAT
		Pork F	ACC ATT GAC TGA ATA GCA CCT
3	ß-Actin	Sus1 F	CGA GAG GCT GCC GTA AAG G
	Gen	Sus1 R	TGC AAG GAA CAC GGC TAA GTG
4	Cyt b	cytb1	CCA TCC AAC ATC TCA GCA TGA TGA AA
		cytb2	GCC CCT CAG AAT GAT ATT TGT CCT CA

Electrophoresis

Agarose gel with a concentration of 2% was prepared by dissolving 8 grams of agarose powder in 200 ml of 1x TAE buffer solution, heated for 1 minute until the agarose dissolved and the solution

was clear. Then 10 µl of safe diamond was added after being warm and homogenized. Then pour it into the caster gel that has been inserted by the comb. Mixing the sample with 1 µl loading dye was carried out using parafilm. The sample DNA used in electrophoresis was 5 µl. Electrophoresis tool running at a voltage of 50 volts for 120 minutes.

The agarose gel electrophoresis result was visualized using UV transiluminator. The UV transiluminator is turned on and the DNA bands will glow when exposed to UV light. This luminescence can be documented with the Enduro GDS-1302 connected to the camera so that the image captured by the camera is then stored on the computer

Results And Discussion

Results of DNA Isolation

The first laboratory-scale process carried out is DNA isolation. DNA isolation of lipstick samples has a high level of difficulty considering the complex composition of the lipsticks. Materials that may contain pig DNA in the lipstick composition include glycerin and oil. This makes pig DNA difficult to detect considering the presence of DNA in the material only as a residue. In the DNA isolation process, lipstick samples were very difficult to homogenize and centrifuge. This can be seen in Figure 1. Low purity results in the sample can reduce the quality of DNA and interfere with the success of analysis at a later stage (Abdullah *et al*., 2019).

Figure 1. The results of the DNA isolation process of cosmetic samples after being centrifuged (left) and after homogeneous (right)

It appears that the DNA isolation process using the Promega kit experienced difficulties in the process of sample homogenization. Furthermore, the results of DNA isolation were quantified. The results of quantitative analysis of genomic DNA isolates can be determined for their quality based on concentration and purity using a Biochrom Biodrop-DUO spectrophotometer. The purity of the DNA isolates can be seen from the absorbance ratio A260/A280. Table 2. Below shows the average DNA purity values obtained from 5 cosmetic samples ranging from 0.479-1,000. These results indicate the results of genomic DNA isolates are still low. According to Wasdili and Gartinah (2018), good DNA quality is when it has a purity level in the range of 1.8 - 2.0 from the absorbance ratio at the measurement of wavelengths of 260 nm and 280 nm. According to Abdullah *et al* (2020), if the A260/A280 ratio shows a result of less than 1.8, it indicates that there are contaminants in the form of proteins and polysaccharides. Meanwhile, if the A260/A280 ratio shows a result of more than 2.0, then the DNA isolate indicates a contaminant in the form of RNA.

Table 2. Purity and Concentration of Genomic DNA Isolation

No	Sampel	Wavelength			Concentration (µg/ml)	Ratio
		A_{230}	A_{260}	A_{280}		
1.	Sampel A	0,012	0,010	0,010	0,220	1,000
2.	Sampel B	0,008	0,008	0,008	0,224	1,000
3.	Sampel C	0,012	0,006	0,007	0,136	0,731
4.	Sampel D	0,012	0,008	0,008	0,224	1,000
5.	Sampel E	0,010	0,005	0,007	0,092	0,479

The result of the low purity of the DNA isolate occurs because the lipstick product has undergone processing processes such as heating and the addition of other ingredients, making it difficult to separate in the DNA isolation process. The purity is

also low considering that the DNA content in this lipstick sample is in the form of residue.

PCR Results

The PCR results were then electrophoresed in the agarose gel stationary phase. The PCR gel documentation of the lipstick samples showed that of the 5 samples that were PCR run using 4 combinations of primers, none of the samples indicated any pig DNA contaminants. This is shown in the absence of DNA bands in samples A, B, C, D, and E, using a combination of primary pairs 1, 2, 3, and 4. The results are shown in Figure 2 below.

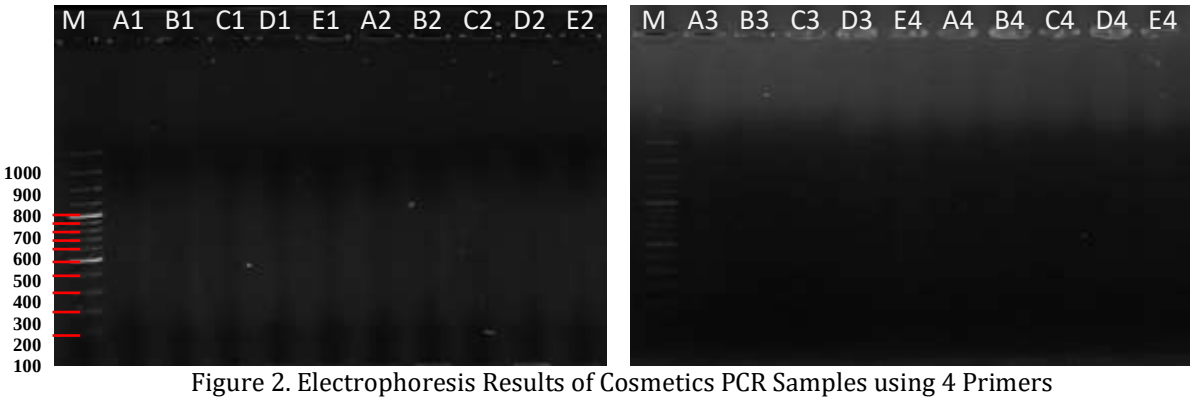

Figure 2. Electrophoresis Results of Cosmetics PCR Samples using 4 Primers
Note: A-E: Sample Name; 1-4: Primary Combinations (Table 1)

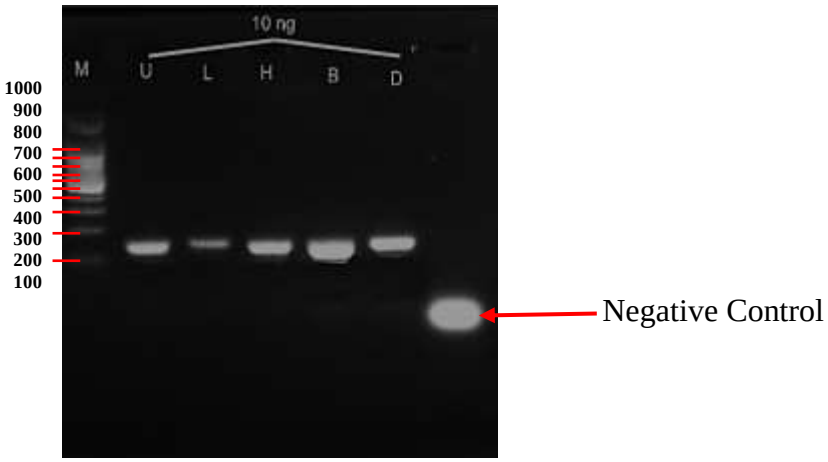

Figure 3. Positive Control: U: Pig intestine; L: Pig Fat; H: Pig Heart; B: Pig Blood; D: Pork; Shows DNA bands of ± 149 bp, using Primer 1 in table 1. The negative control used was beef meat samples.

In positive control with multiple samples of pigs used, DNA bands appear on primer 1. Researchers recommend that they be able to carry out further tests related to the results obtained in this study, given that there are many factors that could be the reason why DNA bands are not produced. These recommendations include: using a different DNA isolation method and continued with the Gold Standard DNA-based testing, namely Real Time PCR. The next recommendation is to use other methods that are not DNA-based, such as fatty acid-based methods. Other methods can be GC-MS, GC-MS-MS.

Conclusion

The results showed that of the 5 samples tested by PCR using 5 kinds of primer combination, none of the samples were suspected to contain pork DNA. DNA isolation is the most difficult step in the lipstick sample detection process. Even though the detection result is negative, it is necessary to carry out further tests which become the Gold Standard of DNA-based testing using Real Time PCR.

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