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ARTICLE

Effect of DNA extraction methods on the detection of porcine ingredients in halal cosmetics using real-time PCR

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Abstract In recent years, halal cosmetics have attracted considerable attention worldwide. We developed a real-time PCR assay based on the mitochondrial gene *ndh5* for rapid detection of porcine ingredients in halal cosmetic products. We also compared several DNA extraction methods for the most efficient approach in different types of cosmetics. Porcine template DNA was spiked into three types of cosmetics (liquid-type and powder-type mask packs, and cream) and extracted with five commercial DNA extraction kits and the CTAB method. The extraction efficiency of each method was evaluated by determining the detection limits of real-time PCR assay. The lowest detection limit of real-time PCR for each cosmetic product was as follows: 2.28×10^0 copies for liquid-type mask pack when the Power Prep™ DNA extraction kit and TIANamp Genomic DNA kit were used, 2.28×10^1 copies for powder-type mask pack when QIAamp DNA stool mini kit and the Power Prep™ DNA extraction kit were used, and 2.28×10^0 copies for cream when the Power Prep™ DNA extraction kit was used. The pig-specific real-time PCR assay facilitated the detection of trace amounts of the template DNA in cosmetics, and an appropriate DNA extraction method was used depending on the type of cosmetics.

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Introduction

With the increase in Muslim population, halal industries are growing rapidly. Halal means 'allowed' according to Islamic law; conversely, haram means 'forbidden' [1]. Therefore, haram elements derived from sources such as pig, dog, animal blood, alcohol, etc., are forbidden during the manufacture of halal products [2]. Currently, halal food is the largest proportion in the global halal market, and cosmetics, medicines, fashion, and tourism are also emerging as major sectors [1]. In particular, the halal cosmetics market is estimated at $56 billion in 2015, accounting for 7% of the global cosmetics market. The halal cosmetics market is expected to reach $81 billion by 2021 [3].

Halal certification is required for a product to enter the halal market. Each product must go through a strict qualification process to ensure that it does not contain any haram elements [4]. Various analytical methods can be used to identify haram elements in halal products. In the case of halal foods, electric nose, gas chromatography, HPLC, NMR spectroscopy, and polymerase chain reaction (PCR) are used to detect hidden animal ingredients such as fats and proteins derived from pig [5–9]. In a few cosmetics, ingredients derived from pigs such as fatty acids, glycerin, and collagen are used in the production of body lotions, creams, and mask packs. As mentioned above, the method of detection of haram components in halal foods may be applicable to halal cosmetics. However, it is not well known.

In general, the detection of specific components in the final product may be limited depending on the analytical method used, as the various components can be modified or degraded by thermal, physical, or chemical treatment during the manufacture. DNA is a relatively stable material for a variety of physical and chemical treatments during the food manufacturing process. Biological extracts added to food or cosmetics are usually not purified to a very high degree due to the high cost involved, and the products are likely to be contaminated with the species-specific DNA.

PCR is a rapid, accurate, and highly sensitive method that can selectively amplify a small amount of target DNA present in a product using species-specific primers [10–12]. In addition, real-time PCR (rt-PCR) is faster, more sensitive, more accurate, and quantitative when compared with PCR because of the additional fluorescent probe [13–15]. Therefore, the rt-PCR method appears to be effective for the detection of pig components in cosmetics. Although PCR is a reliable analytical method, its results can be influenced by the quality of the DNA used [16]. DNA quality is a critical factor in PCR because PCR inhibitors such as metal ions, polysaccharides, polyphenols, and detergents, which may be present in the sample, can affect the amplification of target DNA [17, 18]. Therefore, the preferred DNA extraction method should be simple and rapid and minimize PCR inhibition [19]. Although several studies have compared the efficiency of DNA extraction from a variety of foods [20–22], few studies have examined the efficiency of DNA extraction kits used in the cosmetics industry.

Among the different types of cosmetics, the demand for mask packs, which can be used for cleansing and moisturizing, is increasing worldwide. In addition, cream is one of the basic cosmetic products with a high demand. Therefore, in this study, we developed a pig-specific rt-PCR assay to detect porcine DNA in halal cosmetics. We also compared the performance of various DNA extraction methods by measuring the detection limit of rt-PCR.

Materials and methods

Meats, vegetables, bacterial strains, and cosmetic samples

Pork (*Sus scrofa domesticus*), other meats (chicken, cow, deer, dog, duck, goat, horse, and sheep), and vegetables (carrot, ginger, lettuce, peanut, potato, spinach, soybean, rice, and wheat) used in this study were purchased from local supermarkets in Seoul, Korea. Other meats except pork, vegetables and bacterial strains (*Escherichia coli*, *Bacillus cereus*, *Salmonella enterica*, and *Staphylococcus aureus*) were used as the PCR-negative controls. Cosmetic samples (cream, liquid-type and powder-type mask packs) were obtained from a cosmetic company (Chemland, Gunpo-si, Korea). In addition, several halal-certified and general cosmetics were purchased from a departmental store in Seoul, Korea for field tests.

Chromosomal DNA isolation

Chromosomal DNAs were purified from meat and vegetable samples (about 100 mg) using a Power Prep™ DNA extraction from food and feed kit (Kogenebiotech, Seoul, Korea) according to the manufacturer's instructions as briefly described below. A crushed sample (about 100 mg) was lysed with 400 µL of Lysis buffer A and 40 µL of buffer B for 1 h at 65 °C, followed by chloroform extraction. The sample was centrifuged at 12,000 rpm for 10 min. The supernatant was mixed with 200 µL of Binding buffer and 200 µL of isopropanol. The sample mixture was passed through the column, followed by two washes with 75% EtOH. The DNA was eluted with 100 µL of sterilized distilled water, and the appropriate amount of DNA was used for PCR amplification. All bacterial strains were grown in 5 mL of Luria–Bertani broth at 37 °C. The bacterial DNA was isolated from 5 mL of overnight cultures using the same DNA extraction kit. Purified DNA was recovered in 50–100 µL of sterilized distilled water.

Primer and probe design

The primers and probe used are shown in Table 1. The *ndh5* gene of mitochondrial DNA (GenBank accession number AP003428.1) was used to design pig-specific primers and probe. Comparison of the nucleotide sequences of mitochondrial *ndh5* genes available in the GenBank was used to design the Sus2 and Sus NDH5 primer sets. The PCR amplification of porcine chromosomal DNA with the Sus2 primer set yielded a 403-bp DNA fragment, which was used as porcine template DNA in all spiking experiments. Further, the Sus NDH5 primer set was designed to amplify a 139-bp DNA fragment from the 403-bp template DNA for the detection of porcine DNA in cosmetics. The 5′ and 3′ ends of the TaqMan probe were labeled with 6-carboxyfluorescein and black hole quencher 1, respectively.

Porcine template DNA preparation

A 403-bp porcine template DNA was amplified by PCR from porcine chromosomal DNA with the Sus2 primer set. Amplified DNA fragments were recovered from agarose gel using the PCR clean-up gel extraction kit. The concentration of the purified template DNA was 50 ng/µL measured with UV spectrophotometry. The copy number

Table 1 Oligonucleotide sequences of primers and probe

Primer name	Nucleotide sequence	Amplicon size (bp)
Sus2	F: CCC ATT CGC CTC ACT CAC A	403
	R: GTT GTT GGC GGT TAC GAG GA	
Sus NDH5	F: GCC TCA CTC ACA TTA ACC ACA CT	139
	R: AGG GGA CTA GGC TGA GAG TGA A	
	Probe: GGC GTA GGA TAY CCT CGT TTT TAC GT	

of the template DNA was 2.28×10^{11} copies/μL when calculated using Whelan's formula [23].

DNA extraction from spiked cosmetic samples

The CTAB method and five commercial DNA extraction kits [Nucleo spin food kit (Macherey-Nagal, Germany), Power Prep™ DNA extraction from food and feed kit (KogeneBiotech), QIAamp DNA stool mini kit (Qiagen, Hilden, Germany), TIANamp Genomic DNA kit (Tiangen Biotech, Beijing, China), and Wizard Genomic DNA purification kit (Promega, Madison, WI, USA)] were used to extract the spiked porcine template DNA from cosmetic samples. After mixing 90 μL of cosmetic sample with 10 μL of diluted template DNA, the DNA was extracted using six different methods. All DNA extractions from the spiked samples were carried out according to the manufacturer's instructions. Each purified DNA sample was recovered in 50 μL of sterilized distilled water.

PCR and real-time PCR

PCR was conducted with aliquots of 25 μL, each containing 1 μL of chromosomal DNA, 2.5 μL of 10 × Taq buffer, 0.5 μL of dNTP stock solution (containing 10 mM of each dNTP), 0.125 μL of Taq polymerase (SolGent Co., Daejeon, Korea), 10 pmol of each primer, and deionized water. The PCR was conducted using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following program: 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by 5 min at 72 °C. The PCR product (5 μL) was loaded on a 1.5% agarose gel containing ethidium bromide and visualized under UV illumination.

Real-time PCR was carried out with aliquots of 20 μL, each containing 1 μL of template DNA, 10 μL of 2 × TaqMan Master Mix, 10 pmol of each primer, 2.5 pmol of the probe, and deionized water. The real-time PCR was run on StepOnePlus Real-Time PCR System with the following program: 1 cycle of 2 min at 50 °C and 10 min at 95 °C, 50 cycles of 15 s at 95 °C, and 90 s at 60 °C. The standard curve for porcine DNA was generated with known copies of the template DNA ranging from 2.28×10^0 copies/tube to 2.28×10^{10} copies/tube. The threshold cycle (Ct) values were plotted against the corresponding 10-fold serial dilutions of the template DNA. All PCRs and real-time PCRs were conducted in triplicate.

Results and discussion

Specificity of the pig-specific real-time PCR

Mitochondrial DNA has been widely used to identify species because of its high copy number in animal cells and well-conserved nucleotide sequence [24, 25]. In this study, the mitochondrial *ndh5* gene encoding NADH dehydrogenase subunit 5 was selected as a target for porcine DNA detection based on the nucleotide sequence differences of the mitochondrial *ndh5* genes with other animals (data not shown).

To investigate the specificity of the Sus NDH5 primer set for mitochondrial *ndh5* gene of pig compared with eight other meats, nine vegetables, and four bacterial strains, rt-PCR was performed in triplicate using chromosomal DNA isolated from each sample. No fluorescent signals were observed in the tested samples except pig DNA even after 40 cycles (Table 2). This result indicates that the pig-specific rt-PCR assay facilitates the detection of pig ingredients in halal cosmetics.

Detection limit of the real-time PCR assay

Real-time PCR is widely used to detect and quantify trace amounts of DNA within complex food products [26–28]. To determine the copy number of porcine template DNA that can be detected by rt-PCR, a standard curve was prepared using tenfold serially diluted DNA, ranging from 2.28×10^{10} copies/tube to 2.28×10^0 copies/tube (Fig. 1). The correlation coefficient (R^2) demonstrated the high linearity of the standard curve (Fig. 1A). The Ct value was 39.95 at 2.28×10^0 copies, the smallest amount of template DNA. Generally, a high threshold cycle (Ct) value may be considered as false-positive because of amplification or fluorescence artifacts during real-time amplification [29]. To confirm whether this Ct value was valid, agarose gel electrophoresis was performed to determine the formation of the correct amplicon. As shown in Fig. 1B, the

Table 2 Pig-specific real-time PCR of non-target DNAs

Samples		PCR
Animals	Pig (positive control)	20.46 ± 0.52[a]
	Cow	No Ct
	Chicken	No Ct
	Sheep	No Ct
	Duck	No Ct
	Horse	No Ct
	Dog	No Ct
	Deer	No Ct
	Goat	No Ct
Plants	Potato	No Ct
	Lettuce	No Ct
	Spinach	No Ct
	Peanut	No Ct
	Soybean	No Ct
	Carrot	No Ct
	Ginger	No Ct
	Rice	No Ct
	Wheat	No Ct
Bacteria	*Escherichia coli* ATCC27325	No Ct
	Bacillus cereus ATCC21772	No Ct
	Staphylococcus aureus ATCC25923	No Ct
	Salmonella enterica ACTC13314	No Ct

[a]The threshold cycle (Ct) values are the mean ± SD of three independent experiments

139-bp DNA fragment was amplified, and rt-PCR allowed the detection of the template DNA as low as 2.28×10^0 copies.

Effect of DNA extraction methods on real-time PCR amplification of the spiked porcine DNA

Various ingredients such as alcohols, fats, pectin, and detergents, which may be present in cosmetics, may interfere with PCR [30–33]. Therefore, it is crucial to select the appropriate DNA extraction method to exclude these PCR inhibitors. Although a suitable DNA extraction method and extraction efficiency for cosmetics are not well known, the differences in extraction efficiency depend on the type of food. The CTAB method has demonstrated high extraction efficiency for chocolates and biscuit products [16], whereas the Nucleo spin food kit has high extraction efficiency for protein-rich foods such as soybean flour [21]. In addition, the QIAamp DNA stool mini kit is highly efficient for extracting DNA from vegetative oil [22].

In this study, the porcine template DNA was extracted from spiked cosmetic samples (cream, liquid-type mask pack, and powder-type mask pack) using six different methods, and rt-PCR was used to determine the detection limit corresponding to each extraction method. Initially, to detect porcine DNA in the cosmetics before spiking, DNA was extracted from liquid-type mask pack, powder-type mask pack, and cream using the Power Prep™ DNA extraction kit. Real-time PCR was conducted using the Sus NDH5 primer set and probe for each extracted DNA. Real-time PCR results showed that porcine DNA was not present in the three cosmetics tested (data not shown).

The three cosmetics were artificially spiked with stepwise diluted porcine template DNA, and the DNA was extracted using six different DNA extraction methods. To compare the detection limits of the assay for cosmetics, rt-PCR was performed on spiked porcine DNA extracted from three types of cosmetics using six different methods (Table 3). For liquid-type mask pack, rt-PCR enabled detection of the template DNA as low as 2.28×10^0 copies using the Power Prep™ DNA extraction kit and TIANamp Genomic DNA kit, with Ct values of 38.51 and 38.71, respectively. The rt-PCR sensitivity of the Power Prep™ DNA extraction kit and TIANamp Genomic DNA Kit was 10–100 times greater than that of other extraction methods. For powder-type mask pack, rt-PCR detected DNA as low

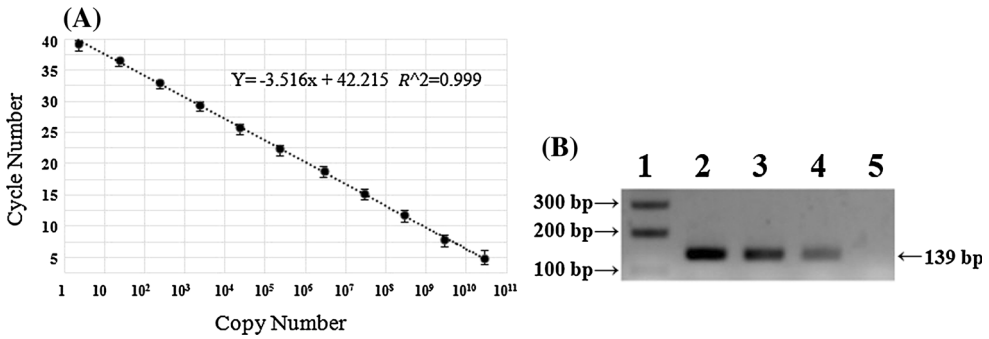

Fig. 1 (**A**) Standard curve generated by real-time PCR using known amounts of template DNA and (**B**) agarose gel electrophoresis of real-time PCR products. Real-time PCR was performed with serially diluted (tenfold) template DNA ranging from 2.28×10^{10} copies/tube to 2.28×10^{10} copies/tube. Negative template control consisted of the reaction mixture without template DNA. Lane 1: 100-bp ladder. Lanes 2, 3, and 4: samples containing template DNA (2.28×10^2, 2.28×10^1, and 2.28×10^0 copies/tube, respectively). Lane 5: negative template control

Table 3 Detection limits of real-time PCR assay for spiked porcine DNA in cosmetic samples

Cosmetics	Extraction methods	Threshold cycle (Ct)				
		2.28×10^4 (copies/tube)	2.28×10^3 (copies/tube)	2.28×10^2 (copies/tube)	2.28×10^1 (copies/tube)	2.28×10^0 (copies/tube)
Liquid-type mask pack	CTAB	27.34 ± 0.60[a]	31.18 ± 0.55	35.06 ± 0.21	No Ct	No Ct
	Power Prep™ DNA extraction kit	24.28 ± 0.19	27.44 ± 0.39	30.97 ± 0.15	35.21 ± 0.21	38.51 ± 0.30
	QIAamp DNA stool mini kit	26.16 ± 0.16	30.49 ± 0.56	34.06 ± 0.15	38.37 ± 0.23	No Ct
	Wizard genomic DNA purification kit	30.56 ± 0.47	33.64 ± 0.33	37.76 ± 0.79	No Ct	No Ct
	TIANamp genomic DNA Kit	24.99 ± 0.21	28.19 ± 0.11	32.07 ± 0.41	35.77 ± 0.49	38.71 ± 0.61
	Nucleo spin food kit	27.19 ± 0.21	31.00 ± 0.11	35.22 ± 0.24	38.94 ± 0.21	No Ct
Powder-type mask pack	CTAB	30.11 ± 0.11	33.86 ± 0.17	38.13 ± 0.14	No Ct	No Ct
	Power Prep™ DNA extraction kit	27.56 ± 0.24	31.94 ± 0.08	35.48 ± 0.37	39.07 ± 0.09	No Ct
	QIAamp DNA stool mini kit	26.63 ± 0.50	29.75 ± 0.67	34.14 ± 0.86	37.63 ± 0.97	No Ct
	Wizard genomic DNA purification Kit	34.14 ± 0.34	38.75 ± 0.40	No Ct	No Ct	No Ct
	TIANamp genomic DNA Kit	30.59 ± 1.28	34.04 ± 1.26	38.42 ± 1.56	No Ct	No Ct
	Nucleo spin food kit	30.40 ± 0.68	33.67 ± 0.22	36.73 ± 0.34	No Ct	No Ct
Cream	CTAB	30.57 ± 0.72	34.69 ± 0.09	38.52 ± 0.52	No Ct	No Ct
	Power Prep™ DNA extraction kit	26.41 ± 0.28	30.16 ± 0.23	34.03 ± 0.27	36.78 ± 0.34	39.07 ± 0.07
	QIAamp DNA stool mini kit	30.97 ± 0.70	34.35 ± 1.22	No Ct	No Ct	No Ct
	Wizard genomic DNA purification Kit	29.96 ± 0.16	34.44 ± 0.38	38.7 ± 0.33	No Ct	No Ct
	TIANamp Genomic DNA Kit	29.52 ± 0.98	33.50 ± 1.20	38.47 ± 1.12	No Ct	No Ct
	Nucleo spin food kit	29.02 ± 0.09	33.15 ± 0.20	36.8 ± 0.33	No Ct	No Ct

[a]The threshold cycle (Ct) values are the mean ± SD of three independent experiments

as 2.28×10^1 copies using the QIAamp DNA stool mini kit and Power Prep™ DNA extraction kit, with Ct values of 37.63 and 39.07, respectively. The rt-PCR detection limit for both kits was 10–100 times higher than the other extraction methods. In the case of cream, rt-PCR detected DNA as low as 2.28×10^0 copies with the Power Prep™ DNA extraction kit alone, and the Ct value was 39.07. The rt-PCR detection limit for the Power Prep™ DNA extraction kit was 100–1000 times higher than the other extraction methods. Cream is mainly composed of lipids such as oils or waxes [34, 35]. Hexane or chloroform treatment minimizes PCR inhibition during DNA extraction from lipid-rich cosmetics [36, 37]. However, the use of organic solvents in the extraction process is tedious and cumbersome; thus, most commercial kits do not use organic solvents. Therefore, the Power Prep™ DNA extraction kit, which uses chloroform during DNA extraction process, may produce optimal results in rt-PCR assays.

Testing of halal cosmetics

In order to detect the presence of pig-derived ingredients in commercial halal cosmetics, six types of halal cosmetics and nine types of general cosmetics (five liquid-type mask packs, five powder-type mask packs, and five creams) were tested. Furthermore, real-time PCR assay was performed using DNA extracted from each cosmetic product with the Power Prep™ DNA extraction kit (Table 4). All of the 15 products tested by rt-PCR were negative, indicating the absence of porcine DNA.

Table 4 Field test of halal cosmetics using real-time PCR assay

Cosmetics	Sample number	Threshold cycle (Ct)
Liquid-type mask packs	1[a], 2[a], 3[a], 4, and 5	No Ct
Powder-type mask packs	1, 2, 3, 4, and 5	No Ct
Cream	1, 2, 3, 4, and 5	No Ct
Positive control (2.28×10^6 copies/tube)		19.11
No template control		No Ct

[a]Cosmetic products were certified by Jabatan Kemajuan Islam Malaysia (JAKIM)

In summary, the real-time PCR assay based on the mitochondrial gene *ndh5* facilitated the detection of trace amounts of the porcine template DNA in cosmetics. This study also showed that the extraction of porcine DNA from cosmetics may depend on the type of cosmetics. Overall, the Power Prep™ DNA extraction kit was the most suitable for isolation of DNA from the tested cosmetics. This pig-specific real-time PCR assay was also applicable for the detection of hidden porcine ingredients in food products or identification of food fraud.

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