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 × 10⁰ copies for liquid-type mask pack when the Power Prep™ DNA extraction kit and TIANamp Genomic DNA kit were used, 2.28 × 10¹ copies for powder-type mask pack when QIAamp DNA stool mini kit and the Power Prep™ DNA extraction kit were used, and 2.28 × 10⁰ 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.

Keywords DNA extraction methods · Halal cosmetics · Pig · Real-time PCR

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 PrepTM 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 ‘TagMan 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
of the template DNA was $2.28 \times 10^{11}$ 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 x 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 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.

**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 $2.28 \times 10^{10}$ copies/tube to $2.28 \times 10^{0}$ 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 $2.28 \times 10^{0}$ 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

| 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 GCC TGA GAG TGA A |
| Probe       | GGC GTA GGA TAY CCT CGT TTT TAC GT |
139-bp DNA fragment was amplified, and rt-PCR allowed the detection of the template DNA as low as $2.28 \times 10^9$ 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 PrepTM 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 step-wise 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 $2.28 \times 10^6$ copies using the Power PrepTM 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
as $2.28 \times 10^4$ 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 $2.28 \times 10^0$ 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 3 Detection limits of real-time PCR assay for spiked porcine DNA in cosmetic samples

| Cosmetics       | Extraction methods                  | Threshold cycle (Ct) |
|-----------------|-------------------------------------|----------------------|
|                 |                                     | $2.28 \times 10^8$ (copies/tube) | $2.28 \times 10^7$ (copies/tube) | $2.28 \times 10^6$ (copies/tube) | $2.28 \times 10^5$ (copies/tube) | $2.28 \times 10^4$ (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             |
| Powder-type mask pack | CTAB                               | 27.19 ± 0.21         | 31.00 ± 0.11               | 35.22 ± 0.24               | 38.94 ± 0.21             | No Ct                   |
|                  | Power Prep™ DNA extraction kit      | 30.11 ± 0.11         | 33.86 ± 0.17               | 38.13 ± 0.14               | No Ct                   | No Ct                   |
|                  | QIAamp DNA stool mini kit          | 27.56 ± 0.24         | 31.94 ± 0.08               | 35.48 ± 0.37               | 39.07 ± 0.09             | No Ct                   |
|                  | Wizard genomic DNA purification Kit | 26.63 ± 0.50         | 29.75 ± 0.67               | 34.14 ± 0.86               | 37.63 ± 0.97             | No Ct                   |
|                  | TIANamp genomic DNA Kit            | 34.14 ± 0.34         | 38.75 ± 0.40               | No Ct                     | No Ct                   | No Ct                   |
| Powder-type mask pack | CTAB                               | 30.59 ± 1.28         | 34.04 ± 1.26               | 38.42 ± 1.56               | No Ct                   | No Ct                   |
|                  | Power Prep™ DNA extraction kit      | 30.40 ± 0.68         | 33.67 ± 0.22               | 36.73 ± 0.34               | No Ct                   | No Ct                   |
|                  | QIAamp DNA stool mini kit          | 30.57 ± 0.72         | 34.69 ± 0.09               | 38.52 ± 0.52               | No Ct                   | No Ct                   |
|                  | Wizard genomic DNA purification Kit | 26.41 ± 0.28         | 30.16 ± 0.23               | 34.03 ± 0.27               | 36.78 ± 0.34             | 39.07 ± 0.07             |
| Powder-type mask pack | CTAB                               | 30.97 ± 0.70         | 34.35 ± 1.22               | No Ct                     | No Ct                   | No Ct                   |
|                  | Power Prep™ DNA extraction kit      | 30.96 ± 0.16         | 34.44 ± 0.38               | 38.7 ± 0.33                | No Ct                   | No Ct                   |
| Powder-type mask pack | QIAamp DNA stool mini kit          | 29.52 ± 0.98         | 33.50 ± 1.20               | 38.47 ± 1.12               | No Ct                   | No Ct                   |
|                  | Wizard genomic DNA purification Kit | 29.02 ± 0.09         | 33.15 ± 0.20               | 36.8 ± 0.33                | No Ct                   | No Ct                   |
|                  | Nucleo spin food kit               | 29.96 ± 0.09         | 33.15 ± 0.20               | 36.8 ± 0.33                | No Ct                   | No Ct                   |

^aThe threshold cycle (Ct) values are the mean ± SD of three independent experiments.
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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**Table 4** Field test of halal cosmetics using real-time PCR assay

| Cosmetics                        | Sample number | Threshold cycle (Ct) |
|----------------------------------|---------------|----------------------|
| Liquid-type mask packs           | 1<sup>a</sup>, 2<sup>a</sup>, 3<sup>a</sup>, 4<sup>a</sup>, 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<sup>6</sup> copies/tube) | 19.11 |
| No template control             |               | No Ct |

<sup>a</sup>Cosmetic products were certified by Jabatan Kemajuan Islam Malaysia (JAKIM)
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