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Analytical Methods

Specific PCR assays to determine bovine, porcine, fish and plant origin of gelatin capsules of dietary supplements

Jae-Hwang Lee [b,1], Mi-Ra Kim [a,1], Cheon-Ho Jo [a], Yoo-Kyung Jung [a], Kisung Kwon [a], Tae Sun Kang [a,*]

[a] New Hazardous Substance Team, Food Safety Evaluation Department, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety, 187 Osongsaengmyeong 2-ro, Osong-eup, Heungdeok-gu, Cheongju-si, Chungcheongbuk-do 28159, South Korea

[b] Department of Food Science, Graduate School, Kyung Hee University, Yongin 17104, Republic of Korea

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ABSTRACT

Gelatin, a purified protein derived mostly from pig skin and bovine tissue, is used widely in both food and pharmaceutical industries. Here, to determine the species of origin of capsule gelatin, we developed a sensitive and reliable test using the polymerase chain reaction (PCR) method, which included 1) species-specific or universal primer sets, designed to detect short 16S ribosomal RNA (rRNA) gene sequences from cow, pig, and fish (tilapia) as well as genes encoding the large subunit of plant ribulose-1,5-bisphosphate carboxylase oxygenase and 2) species-specific PCR coupled with whole-genome amplification. This method was used to verify manufacturing label claims of 28 gelatin capsule samples sold as dietary supplements. The results from 27 samples were consistent with gelatin-related information on the manufacturer label, while one sample that mentioned tilapia gelatin was found to contain only bovine DNA. This rapid method can therefore be used to verify the authenticity of gelatin capsules.

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1. Introduction

According to the South Korean Food Code (Article 5), capsules are considered a type of food formulation containing additives such as gelatin, glycerin, and other materials. Gelatin is a major component of capsules and is mostly obtained by hydrolysis of collagen extracted from animal bone, hide, and skin procured from animal slaughterhouses (Karim & Bhat, 2008). In Europe, the majority of edible gelatin is derived from pig skin; however, a significant proportion originates from bovine hide and splits (Tasara, Schumacher, & Stephan, 2005). Gelatin is widely used as a gelling and thickening agent in a variety of foodstuff, including confectionary products and water-based desserts, as well as pharmaceutical medicine capsules. Several steps are involved in the production of gelatin, for example, acidic or basic hydrolysis of connective tissue raw material, high temperature and pressure extraction with water, sterilization, and drying. These processes are not standardized and affect the properties of the final gelatin product. As a result, the proteins and nucleic acids in the final gelatin product are highly degraded and their levels are very low and often variable (Boran & Regenstein, 2010).

Several attempts have been made to identify the origin of gelatin. Fourier transform infrared (FTIR) spectroscopy is a rapid method that differentiates between raw bovine and porcine gelatin based on spectral intensity; however, this method requires high purity of the sample and cannot discriminate within a mixture of raw gelatin (Hashim et al., 2010). High-performance liquid chromatography (HPLC) in conjunction with principal component analysis (PCA) differentiates between raw bovine and porcine gelatins. However, this method also does not identify a mixture of gelatins due to similarities in their chemical properties (Nemati, Oveisi, Abdollahi, & Sabzevari, 2004). Enzyme-linked immunosorbent assay (ELISA) is a sensitive technique for detecting the origin of gelatin based on antibody and antigen reactions; however, due to high homology of collagen sequences among animals, this method is unlikely to be species-specific (Venien & Levieux, 2005). HPLC coupled with mass spectrometry is an alternative that differentiates the origin of gelatin on the basis of marker peptides within collagen sequences (Zhang et al., 2008, 2009). However, marker peptides may also be degraded during manufacturing.

Therefore, DNA-based methods to verify the origin of gelatin materials have been considered a better alternative due to the greater stability of DNA in highly-processed food. In addition, DNA is present in most biological tissues and can be extracted from even a very small amount of sample. Therefore, polymerase chain reaction (PCR)-based methods are an ideal alternative for

* Corresponding author.
E-mail address: missa@korea.kr (T.S. Kang).
[1] These authors contributed equally to this work.

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identifying adulteration of material from other species (Linacero et al., 2016; Lockley & Bardsley, 2000). To date, PCR analyses using species-specific primers have been used for sensitive and specific detection of various meat, seafood, and dairy products (Dalmasso et al., 2004; Fumiere, Dubois, Baeten, von Holst, & Berben, 2006). However, a limited number of studies have been reported regarding identification of the origin of gelatin, gelatin-containing foods, and capsule shells.

In this study, we designed species-specific primer sets for bovine, porcine, and fish (tilapia) and universal primer sets for fish and plants. We optimized species-specific and universal PCR conditions to assess the origin of gelatin capsules. This method was used to verify the manufacturing label claims of 28 commercially available gelatin capsules sold via the internet as health supplement foods.

2. Materials and method

2.1. Samples

For the isolation of DNA, which was used to optimize species-specific PCR conditions, samples of beef, pork, tilapia, and plant material (sweet potato) were purchased from local markets. Standard capsules composed of blending (bovine and porcine) gelatin or gelatin of bovine, porcine, or fish origin, or hydroxypropyl methylcellulose (HPMC) material were obtained from Suheung Capsule Co. Ltd. (Osong, South Korea). A total of 28 different commercially available gelatin capsules, described as health supplements, were purchased via the internet.

2.2. DNA extraction

DNA was extracted from meat and plant samples (100 mg) using either DNeasy® Blood & Tissue or DNeasy® Plant kit (Qiagen, Hilden, Germany), according to the manufacturer instructions, with minor modifications as described below. To isolate DNA from the standard capsules, 200 mg empty capsules were minced using a pair of scissors and DNA was extracted with either the DNeasy® Blood & Tissue or DNeasy® Plant kit (Qiagen). For animal capsules, 200 mg homogenized sample was mixed with 360 μL ATL buffer and 40 μL protease K, and the mixture was incubated at 56 °C until complete lysis. The lysis solution obtained was mixed with 400 μL AL buffer and 400 μL ethanol (96%–100%), and the lysate was transferred into a DNeasy Mini spin column. After centrifugation at 6000×*g* for 1 min, the column was washed twice with washing buffer (AW1 and AW2), and purified DNA was eluted by adding 50 μL Tris-EDTA (TE) buffer. For HPMC capsules, 200 mg homogenized sample was mixed with 800 μL AP1 buffer and 8 μL RNase A, and the mixture was incubated at 65 °C for 20 min. The lysis solution obtained was mixed with 260 μL P3 buffer, and the mixture was incubated in ice for 10 min. After centrifugation at 20,000×*g* for 5 min, the lysate was transferred into a QIAshredder spin column. Flow-through solution was mixed with 1.5 volumes of AW1 buffer and transferred into a DNeasy Mini spin column. After centrifugation at 6000×*g* for 1 min, the column was washed twice with AW2 buffer and purified DNA was eluted by adding 50 μL TE buffer. For DNA extraction from capsules containing commercial health supplements, the contents were removed completely and the empty capsules were washed well with distilled water. DNA was extracted as described above. To confirm the statistical significance of results

2.3. Whole genome amplification

To obtain a larger quantity of DNA from gelatin capsules, DNA extracts were amplified using the GenomePlex® Whole Genome Amplification (WGA) kit (Sigma-Aldrich, St. Louis, USA) according to the manufacturer instructions, purified using AccuPrep® PCR Purification kit (Bioneer, Seoul, South Korea) according to the manufacturer instructions, and quantified using NanoDrop® ND-1000 UV–vis Spectrophotometer (Thermo Fisher Scientific, Delaware, USA). DNA concentration was determined by UV absorbance at 260 nm (1 absorbance unit corresponds to 50 μg/mL dsDNA). The purity of the extract was determined by the ratio of the absorbance at 260 nm to that at 280 nm.

2.4. Target gene selection and oligonucleotide primers

The species-specific primers used in this study targeted bovine (accession No. HM045018), porcine (accession No. GU147934), and tilapia (accession No. NC_013663) mitochondrial 16S rRNA genes (Supplemental Fig. S1A and Table 1). For the identification of fish capsules, a universal primer set was designed based on 16S rRNA genes of sea bass (accession No. GU324142), cod (accession No. GU324163), and yellow-fin tuna (accession No. HM071029) (Supplemental Fig. S1B and Table 1). For the identification of vegetable capsules, a universal primer set was designed based on the chloroplast ribulose 1,5-bisphosphate carboxylase oxygenase large subunit gene (*rbcL*) of potato (accession No. HF572814), sweet potato (accession No. JX139773), and tapioca (accession No. JX139772) (Supplemental Fig. S1C and Table 1). Multiple alignment was constructed from the sequences of 16S rRNA genes or *rbcL* using BioEdit software, version 7.2.2.

2.5. PCR amplification and DNA sequencing

Conventional PCR was conducted in a total volume of 20 μL containing 1–10 ng template DNA, 0.5 μM of each primer, 1× PCR Buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 2.0 mM $MgCl_2$, 1 U rTaq polymerase (TaKaRa Bio Inc., Otsu, Japan), and sterile distilled water. The reactions were performed in a thermal cycler C1000 Touch™ (Bio-Rad Laboratories, Hercules, USA) under conditions described in Table 2. The amplified fragments were analyzed by 2.0% agarose gel electrophoresis. In order to verify the sequences of short-length fragments produced by the species-specific and universal primers, PCR products were eluted from agarose gels and cloned into the pGEMT-easy vector (Promega, Madison, USA). Plasmid DNA was purified using the AccuPrep® PCR Purification kit (Bioneer), and the samples were sent to Bioneer Corp. (Seoul, South Korea) to determine nucleotide sequences.

3. Results and discussion

3.1. Specificity and sensitivity of the PCR assays

In general, gelatins are highly-processed products, resulting in a high degree of degradation of the extracted DNA. Mitochondrial genes such as these coding for 12S rRNA, 16S rRNA, 18S rRNA, cytochrome b, cytochrome oxidase II, and NAD dehydrogenase, are widely used for species identification, mainly due to their high copy number as compared to nuclear DNA (Ballin, Vogensen, & Karlsson, 2009). In addition, amplification of small fragments of the mitochondrial genes is recommended for analysis of degraded DNA extracted from highly-processed food samples. Therefore, in this study, the primers were designed to detect short sequences of 16S rRNA genes, which are highly abundant in cattle, pig, and

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Table 1
Primers used in this study.

Species	Primer	Target gene	Sequence (5′–3′)	Tm (°C)	Size (bp)	Sources
Bovine	SFI11-Cow-F SFI11-Cow-R	*16S rRNA*	TATCTTGAACTAGACCTAGCCCAATG GGTACTTTCTCTATAGCGCCGTAC	56.1 54.6	131	
Porcine	SFI11-Pig-F SFI11-Pig-R	*16S rRNA*	CAACCTTGACTAGAGAGTAAAACC GGTATTGGGCTAGGAGTTTGTT	54.4 57.7	138	
Tilapia	SFI11-Til-F SFI11-Til-R	*16S rRNA*	TTTAAATTCTTTACCCCCATTGGC CTGCTTTTAGGCCCACTAGAACATTAG	58.3 58.9	167	This study
Fish	F16-F1 F16-R1	*16S rRNA*	TAATAAACAAATAAGAGGTCCCG GGAGACAGTTAAGCCCTCGTCAT	51.4 57.4	151	
Plant	PR-F2 PR-R2	*rbcL*	GATTCGCAAATCTTCCAGACG TCTTCTACTGGTACATGGACAACT	56.0 51.3	255	

Table 2
Optimized PCR conditions for the 4 species analyzed in this study.

	Program step	Primers				
		SFI11-Cow-F/ SFI11-Cow-R	SFI11-Pig-F/ SFI11-Pig-R	SFI11-Til-F/ SFI11-Til-R	F16-F1/ F16-R1	PR-F2/ PR-R2
	Initial denaturation	94 °C (10 min)[a]				
Amplification	Denaturation	94 °C (30 s)	94 °C (30 s)	94 °C (30 s)	94 °C (30 s)	94 °C (30 s)
	Annealing	59 °C (10 s)	59 °C (10 s)	60 °C (30 s)	60 °C (10 s)	60 °C (10 s)
	Extension	72 °C (40 s)	72 °C (40 s)	72 °C (30 s)	72 °C (1 min)	72 °C (1 min)
	Cycle number	40	40	40	35	35
	Final extension	72 °C (5 min)[a]				

[a] These conditions were the same for all primers.

capsules. Vegetable capsules are mostly prepared from starch extracted from tapioca, potato, or corn, or from hydroxypropyl methylcellulose (HPMC), a synthetically modified form of cellulose (Stroud, 1996). Considering that various plant materials are used to prepare vegetable capsules, we designed plant universal primers based on the sequences of *rbcL* genes. This gene has been widely used as a marker for polygenetic analysis because it is highly conserved (Olmstead & Reeves, 1995). The identification of bovine, porcine, tilapia, fish, and plant materials was conducted using the species-specific or universal primers listed in Table 1.

The specificity of the designed primers was tested against 4 materials, that is, cattle, pig, tilapia, and plant materials, which are commonly used as raw material for gelatin. As shown in Fig. 1, each pair of primers could only produce the expected fragments (131 bp for cattle, 138 bp for pig, 167 bp for tilapia, 151 bp for fish, and 255 bp for plant) when the corresponding DNA for which they were designed was used as templates, suggesting no cross-reaction among the four materials.

The sensitivity of our method was determined using the DNA extracted from each reference species, with concentrations starting from 10 ng/μL. The detection limit for each species was tested with decreasing concentration of reference DNA. DNA extracts from cattle, pig, tilapia, and sweet potato were diluted from 10^1 to 10^5, corresponding to a DNA concentration of 1–0.0001 ng/μL. The detection limit for bovine and porcine species was 0.001 and 0.01 ng/μL, respectively; however, a higher detection limit (0.1 ng/μL) was observed for tilapia-specific primers (Fig. 2A–C). In the case of the universal primers, the detection limit for fish and plants was 0.01 and 0.0001 ng/μL, respectively (Fig. 2D and E), showing that the fish universal primer set had 10-fold higher sensitivity than the tilapia-specific primer set.

3.2. Whole genome amplification of extracted DNA

Fig. 3B and E, PCR products with very weak intensity were detected by the porcine-specific primer set and the plant universal primer set, likely due to low quantity of initial template DNA. Previous studies have reported that treatment with the WGA kit increases the quantity of DNA extracted from olive oil by 17- to 22-fold (Muzzalupo, Pellegrino, & Perri, 2007). Focke, Haase, and Fischer (2011) reported that the expected PCR products can be detected from WGA-enriched clove and all-spice DNA using species-specific primers.

Therefore, we carried out WGA to increase the quantity of amplifiable DNA and consequently increase the ratio of DNA quantity versus inhibiting substances. DNA was extracted from the 5 standard capsules and the extracted DNA was amplified using the WGA kit. Compared with untreated and WGA-treated DNA, visible DNA fragments ranging from 100 to 1000 bp were obtained with WGA (data not shown). Species-specific PCR was conducted using the WGA products as a template under the optimized conditions. Thus, the expected PCR products for cattle (131 bp), pig (138 bp), tilapia (167 bp), fish (151 bp), and plant (255 bp) were detected only after WGA, by agarose gel electrophoresis (Fig. 3). Interestingly, as shown in Fig. 3E, the plant universal primers produced unexpected PCR products of the same size as that of the plant-specific PCR product (255 bp) from blending (bovine and porcine), bovine, porcine, and fish gelatin capsules after WGA.

To identify the origin of each amplicon, the 4 bands were eluted from the agarose gels, cloned into the pGEMT-easy vector, and sequenced using M13 sequencing primers. The NCBI BLAST database was screened with the sequences of each PCR product as a query using BLASTn. In our BLAST search, all PCR products showed high sequence identities of over 98% to *rbcL* from various plants, including *Raphistemma pulchellum*, *Fallopia convolvulus*, and *Muehlenbeckia platyclada*. In addition, we aligned the sequences of the 4 PCR products and potato *rbcL* that was used to design our plant universal primers, by using the BioEdit software and

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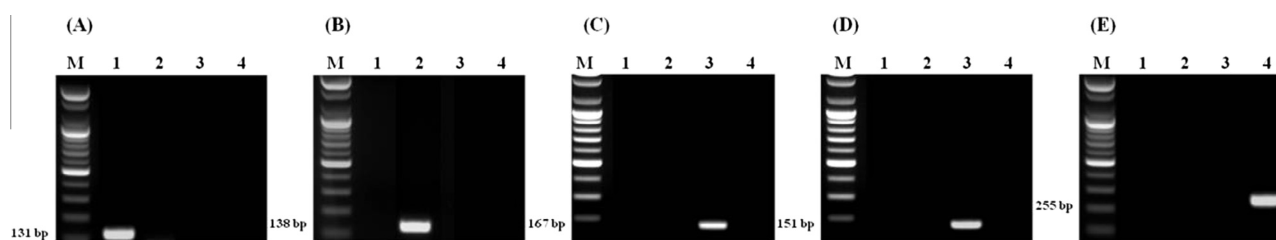

Fig. 1. Selectivity of PCR assays using bovine (A)-, porcine (B)-, and tilapia (C)-specific primers and fish (D) and plant (E) universal primers. M: 100 bp ladder, Lane 1: bovine DNA, Lane 2: porcine DNA, Lane 3: tilapia DNA, and Lane 4: sweet potato DNA.

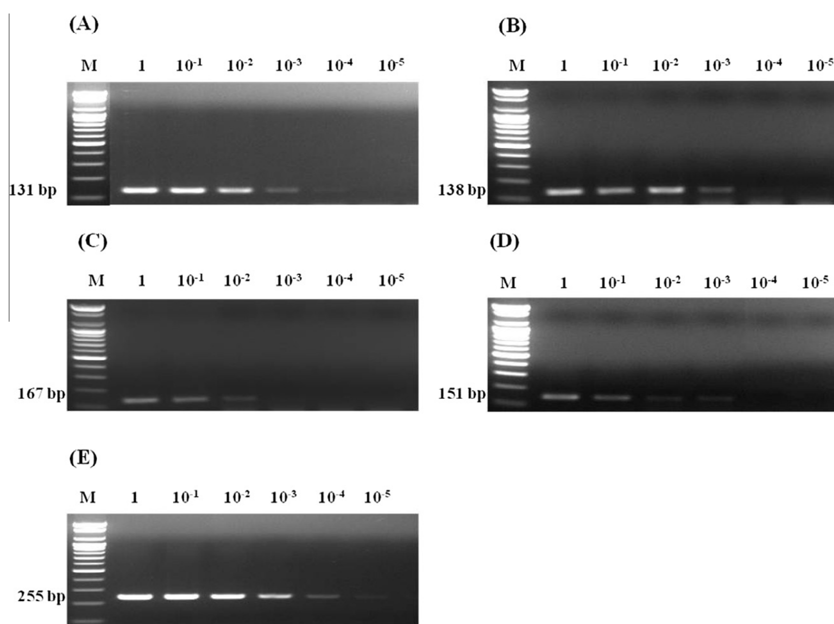

Fig. 2. Detection limit of PCR assays using bovine (A)-, porcine (B)-, and tilapia (C)-specific primers and fish (D) and plant (E) universal primers. Bovine (A), porcine (B), and tilapia (C and D), sweet potato (E) DNA was used as a template for the assays. M: 100 bp ladder, Lane 1: 10 ng/μL DNA, Lane 2: 1 ng/μL DNA, Lane 3: 0.1 ng/μL DNA, Lane 4: 0.01 ng/μL DNA, Lane 5: 0.001 ng/μL DNA, and Lane 6: 0.0001 ng/μL DNA.

was performed with sequences of the 4 PCR products only, suggesting that these sequences could originate from plants other than potato. We also confirmed from the manufacturer (Suheung Capsule Co. Ltd.) that various plant-derived materials such as plasticizers had been added during the manufacturing process in order to produce the gelatin capsule shapes; however, exact amounts or types of plant materials used were not disclosed by the manufacturer as this was proprietary information. Therefore, PCR products as shown in Fig. 3E would originate from plant-derived materials in the gelatin capsules.

3.3. Application of species-specific PCR assays to gelatin capsule samples

As a next step, we used our method to assess the authenticity of gelatin capsules from commercial dietary supplements. The capsules from 28 commercial dietary supplements were subjected to DNA extraction and WGA. The optimized PCR conditions for each species were then applied to the sample extracts for identification of gelatin material and verification of labeling compliance. As shown in Table 3, we found that 25 capsules labeled as gelatin contained bovine material, 11 of which were of the blending type, containing both, bovine and porcine materials and these were also taining marine gelatin showed a positive result. In 1 sample labeled as vegetarian, a positive result was detected by the plant universal PCR assay only. Thus, these results were consistent with the labeling information provided by the manufacturers. However, the plant universal primers also produced positive results from both bovine and blending-type capsules. Thus, to confirm whether these PCR products were obtained from plant-derived material in the gelatin capsules or from the commercial dietary supplements contained therein (e.g., flax oil, L-ornithine, and inositol), we performed PCR assays with DNA extracted from the contents for comparison, with the plant universal primer set. However, a detectable amount of DNA could not be obtained from the dietary supplements (data not shown). It is reported that excipients in dietary supplements can absorb DNA, hampering its extraction and further amplification; therefore, we conducted a spiking assay of the capsule contents with sweet potato DNA (50 ng), using the method previously described by Costa et al. (2015). When compared to the control (sweet potato DNA in water, 5 ng/μL), recovery of the spiked DNA was 40%, 60%, and 76% from flax oil, L-ornithine, and inositol, respectively, resulting in PCR amplification. However, no amplification occurred on using plant universal primers to amplify DNA extracted from the dietary supplements spiked with water (Supplemental Fig. S3). Therefore, our result demonstrated that the PCR products obtained using plant universal primers resulted

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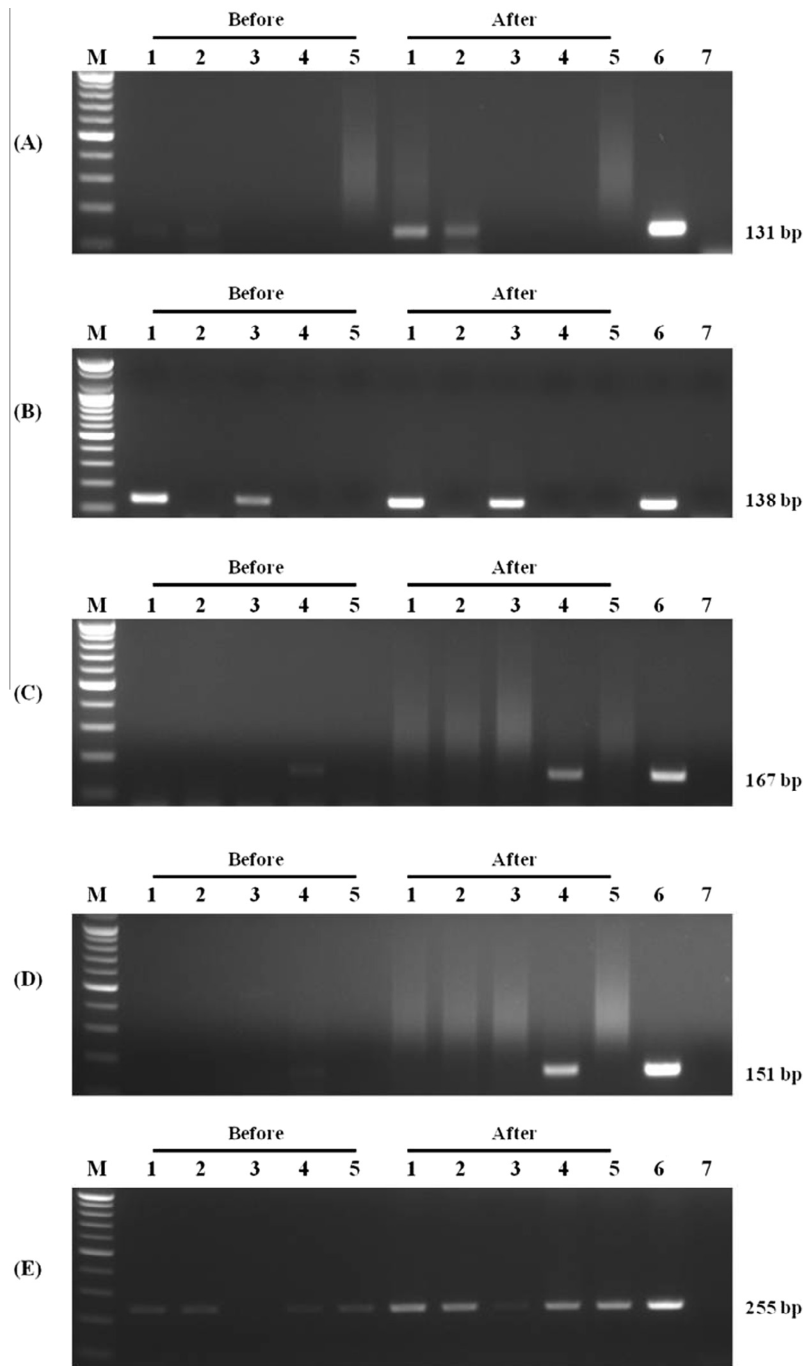

Fig. 3. Effect of whole genome amplification on species-specific PCR. The PCR assays were conducted with bovine (A)-, porcine (B)-, and tilapia (C)-specific primers and fish (D) and plant (E) universal primers and DNA extracted from capsules before and after WGA. M: 100 bp ladder, Lane 1: blending (bovine and porcine) capsule, Lane 2: bovine capsule, Lane 3: porcine capsule, Lane 4: fish capsule, Lane 5: plant (HPMC) capsule, Lane 6: positive control (each corresponding species DNA), Lane 7: negative control.

1 sample that was labeled as tilapia gelatin showed a negative result with the tilapia-specific and fish universal PCR assays; however, bovine DNA was detected for this sample. To further confirm the origin of gelatin from this sample (No. 28), we cloned the PCR product produced by bovine species-specific primers and sequenced it. In our BLAST search, this PCR product showed over 98% sequence identity to 16S rRNA genes of bovine species. Thus, this observation suggested a possibly fraudulent substitution of tilapia gelatin with bovine gelatin.

The issue of gelatin consumption has raised concerns for health form encephalopathy (BSE) has raised concerns about potential risks related to consumption of bovine gelatin (Cai, Gu, Scanlan, Ramatlapeng, & Lively, 2012). Because of religious concerns, Hindu customs do not permit consumption of gelatin of bovine origin, whereas consumption of gelatin of porcine origin is strongly prohibited by Muslim and Jewish kosher dietary laws (Van der Spiegel et al., 2012). As a result, great efforts have been made to replace mammalian gelatin, leading to the development of methods for gelatin extraction and production from fish (Gudmundsson & Hafsteinsson, 1997; Karim & Bhat, 2009). In

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