Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

A Unified Method for Different Placental Products Species Identification

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Submitted: 23-7-2019 Revised: 11-10-2019 Published: 31-03-2020

ABSTRACT

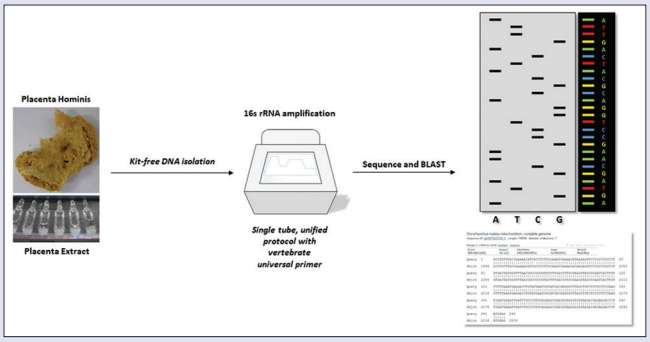
Background: Placentas are widely used in the production of cosmetics, Chinese medicines, and injections. As the esthetic medicine market grows, challenges such as impure preparations and an increasing number of counterfeit products develop, owing to the lack of standard inspection methods and accurate description of the composition of these products. **Objectives:** To develop a universal species identification method for placental products. **Materials and Methods:** Fresh swine, cattle, goat, and frozen human placenta were used as references, while Placenta Hominis and placental extract products from the market as trial samples. Samples were prepared by kit-free DNA isolation, and vertebrate-specific primer sets of mitochondrial 16S and 12S rRNA were used for species-specific region amplification. Direct sequence of amplicons and National Center for Biotechnology Information database comparison was carried for species identification of the testing samples. **Results:** Trail tests had confirmed the usability of this method. Six of seven Placenta Hominis samples were shown to contain human DNA traces, while the seventh showed no DNA signals belonging to any mammals and a paper-like material underneath the vegetal part, instead of membrane-like placental tissue by visual inspection, suggesting the possibility of counterfeit. Eight injectable and cosmetic placental extract products were tested, and none of them contained analyzable DNA or comparable protein fragments except one, which DNA was from rainbow trout (*Oncorhynchus mykiss*) rather than sheep, as per the product claim, and was hence misbranded as per the U.S. Food and Drug Administration definition. **Conclusion:** Our species identification method is easy-to-operate, unified, and resource-saving, which can be applied to different placental products.

Key words: Mitochondrial DNA, Placenta Hominis, placental extract, species identification, vertebrate-specific primers

SUMMARY

In this study, we reported the following achievements:

- Kit-free placental material and placenta extract DNA isolation and a unified polymerase chain reaction condition with minimal laboratory resources requirement
- Great impurity resistance by vertebrate mitochondrial DNA identification in Placenta Hominis
- Species identification capability in injectable placental extract products containing DNA fragments.



Abbreviations used: BLAST: Basic Local Alignment Search Tool; Bp: Base pair; COI: Cytochrome c oxidase subunit I; DNA: Deoxyribonucleic acid; EDTA: Ethylene diamine tetraacetic acid; FDA: U.S. Food and Drug Administration; G: Gram; G: Gravitational force unit; H: Hour; Kb: Kilo base pair; M: Molar; Mg: Milligram; Min: Minute; Ml: Milliliter; mtDNA: mitochondrial DNA; NCBI: National Center for Biotechnology Information; rRNA: Ribosomal ribonucleic acid; PCR: Polymerase chain reaction; S: Second; Seq: Sequence; SDS: Sodium dodecyl sulfate; TE: Tris-EDTA; µl: Microliter; %: Percent.

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DOI: 10.4103/pm.pm_296_19

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INTRODUCTION

Placenta is a temporary supportive organ that connects a developing fetus to the maternal uterine wall and is involved in nutrient uptake, waste elimination, and gas exchange through the mother's bloodstream. After birth, the placenta is expelled from the mother's body. Considerable amounts of nutrients and hormones are retained in the placenta during pregnancy.[1] A wide range of mammals eat their placenta after childbirth that is a phenomenon known as placentophagy.[2,3] Placenta has been used in medicine throughout human history. Placenta Hominis is a traditional Chinese herbal material made using human placenta. The production method involves deep-fried placenta mixed with other herbs, corn, or bean powders and baked at low heat until dry. The placental tissue shrinks into a membrane-like structure covered with vegetable powders.[4] Placenta Hominis has been used both as a lactagogue[5], as an energy supplement and for treating night sweats, impotence, and infertility. In other countries, placentophagy also exists as a folk remedy.[2,3] However, due to limited supply and lack of regulation, extensive use of placental

Cite this article as: Chang TL, Wu SF, Wang DY, Huang CH. A unified method for different placental products species identification. Phcog Mag 2020;16:S8-12.

sources from other species prompts the need for better regulation and product identification.

In modern studies, placental extract products have shown beneficial effects as antioxidants,[6,7] antidepressants,[8] in wound healing,[9] in the treatment of rheumatoid arthritis,[10] in osteoarthritis[11] and arthritis,[12] as pain relievers,[13] biologic fillers,[14] anti-inflammatory substances,[15] and hair growth promoters,[16] among other effects. A wide range of nucleic acids, hormones, and proteins within placenta play important roles in these functions.[1,7,17] Placental extract products have also been widely used in cosmetic products since the early twentieth century.[18,19] Placental extract is produced by homogenization, protein hydrolysis, or supercritical fluid extraction[20] followed by further filtration and purification steps. Different extraction methods yield different compositions of the final products; however, all these products are sold under the same name, "placental extract," confusing the public and posing challenges for the administration. According to the U.S. Food and Drug Administration (FDA) cosmetic handbook, products should not be identified by the name "placental extract" but by a name which describes their composition more accurately.[21] Many of the current products with placental extract contain both purified and unpurified components. Due to limited knowledge of the composition of placental extract and lack of standard inspection protocols, several challenges, such as impurities in placental ingredients and production of counterfeits, exist, leading to limited effectiveness and causing irritation or allergies.

Earlier studies on Placenta Hominis identification were focused on cytochrome c oxidase subunit I (COI) region by barcode[22] or species-specific primer sets.[23] Without specific primer sets separating animal and vegetal species, these approaches require either more delicate sample preparation or separate amplification for every target species, to reduce signal noise due to contamination.

In this study, we developed a kit-free DNA isolation method adopted for raw placenta and placental products followed by a unified polymerase chain reaction (PCR) condition using vertebrate-specific primer sets to amplify part of mitochondrial 12S and 16S rRNA for species identification.[24] This species identification method has high accuracy, is time-consuming and cost-saving, and is a unified procedure. It has the potential to identify components from raw animal material through to end products, thereby providing a powerful tool for setting certification test standards, commodity inspection, and customs examination of animal materials that are imported or exported.

MATERIALS AND METHODS

Placental samples

All fresh placentas except human placenta were obtained from a local farm and slaughterhouse. Human placental samples were purchased from ILSbio (sample number 1-410-810-7506). Placenta Hominis samples were purchased from local Chinese medicine shops. Placental extract product samples (seven injectable and one cosmetic) were purchased directly from the suppliers.

Reagents, bacterial strain, and plasmids

All chemicals were from Merck except agarose from AMRESCO, antibiotics from Sigma, and enzymes from Roche, New England Biolabs, and TAKARA. DNA extraction and PCR clean-up kits were from Viogene. Cloning vector pBluescript II KS(-) from Stratagene was used for PCR product cloning. *Escherichia coli* strain DH5α was used, and standard culture conditions for molecular cloning were followed.[25]

Sample DNA isolation

Total DNA of the raw placenta and Placenta Hominis samples were isolated using the following protocol: 0.1 g of tissue was taken from the test sample. It is important that samples are cut vertically if using Placenta Hominis so that they contain both vegetal and animal parts. Samples were washed and soaked till softened in 10 times volume of the Tris-ethylene diamine tetra acetic acid (TE) buffer containing 0.1% sodium dodecyl sulfate (SDS), crushed into small pieces, and rinsed with TE buffer containing 0.1% SDS at 37°C for 5 min. Samples were vortexed and the supernatant was discarded. The rinsing procedure was repeated four times. The sample was treated with 0.4 mg proteinase E, in 700 ml TE buffer containing 0.1% SDS at 50°C for 8 h. After addition of another 0.4 mg of proteinase E, samples were incubated for another 4 h. For the removal of proteins and digested peptides, 100–200 μl of phenol/chloroform was added and mixed completely, the sample centrifuged at 15,000 g for 10 min, and the upper layer collected into a new Eppendorf tube. This step was repeated until the white interlayer disappeared. DNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 0.65 volume isopropanol. The sample was mixed by inverting the tube 2–3 times and then allowing to stand for 10 min at room temperature (20°C–30°C). The sample was spun at 15,000 g for 10 min, the supernatant was discarded, and the pellet was washed with 70% ethanol (−20°C), air-dried, and then resuspended in sterile water.

For placental extract products, 300 μl of the sample was taken and followed the same protocol starting from phenol/chloroform treatment to the end.

Primer sets

Primers were synthesized by a local biotech company (Genomics). Primer sequences were identical to those in a published study[24] and are listed in Table 1.

Polymerase chain reaction amplification

PCR amplification was carried out using high-fidelity thermostable DNA polymerase (TAKARA) with the following protocol: 98°C initialization for 30 s, 35 amplification cycles of 98°C denaturation for 10 s, 57°C annealing for 15 s, 72°C elongation for 30 s, and 72°C final elongation for 5 min and then held at 4°C. Positive control containing cloned placental 12S and 16S partial region as templates, while negative controls replacing DNA template with ddH_2O were included in each test batch to eliminate contamination due to human handling. Amplified PCR products were checked by gel electrophoresis.

Cloning and sequencing

After PCR amplification, PCR products were sent for sequencing. For positive control preparation, PCR products of partial 12S and 16S regions from placental tissue of reference animal species were cloned into pBluescript II KS(-) vector and transformed into DH5α competent cells. IPTG/X-gal selection single colonies were picked from Luria-Bertani (LB) agar plates with ampicillin and amplified in LB broth.

Table 1: Universal vertebrate primer sequences of 12S and 16S mitochondrial deoxyribonucleic acid

Locus	Primer	Seq (5uenceonu
12S rRNA	12S-1	CCCAAACTGGGATTAGATACCC
	12S-2	GTTTGCTGAAGATGGCGGTA
16S rRNA	16S-1	GCCTGTTTACCAAAAACATCAC
	16S-2	CTCCATAGGGTCTTCTCGTCTT

rRNA: Ribosomal ribonucleic acid; Seq: Sequence

Amplified PCR product-containing plasmids were sequenced, ensuring correct preparation of the positive controls.

RESULTS

Establishment of a method for accurate identification of distinct animal raw placentas

The DNA isolation and unified PCR protocol were tested with fresh placentas from cattle, goat, swine, and frozen human tissue as the standard materials. Three experimental repetitions were done for each species, and the isolated DNA was checked by gel electrophoresis [Figure 1a]. Clear trace of isolated DNA was observed from all samples. PCR was done with two sets of vertebrate-specific mitochondrial DNA (mtDNA) primers, resulted in a clear 200–300 bp amplified band in all samples [Figure 1b]. Each amplicon was sequenced directly, BLAST with National Center for Biotechnology Information database and confirmed to be 100% identical to its related species. This results showing that our unified procedure works with the raw placentas, providing a trustworthy base before we examing placental products.

Applicability of this method to Placenta Hominis

Seven samples of Placenta Hominis were collected from different local Chinese medicine shops. We successfully isolated DNA from all the samples; six out of seven DNA samples were amplified by PCR and sequenced. All of the sequenced samples showed the highest similarity to the human reference sequence. BLAST results of the six samples showed 99.59%–100% identity to human mtDNA with the expect value between $2e^{-121}$ and $4e^{-123}$ for the 16S mtDNA and 99.53%–100% identity with the expect value between $2e^{-105}$ and $5e^{-107}$ for the 12S mtDNA. We inspected the particular sample with no PCR amplification; it is very similar to other Placenta Hominis other than a paper-like material underneath the vegetal part with cellulose-like fibers at the edges, instead of membrane-like placental tissue[4] [Figure 2]. This paper-like membrane layer was not found in other Placenta Hominis samples. We sent this sample for proteomic analysis by Proteomics Research Center of National Yang-Ming University. The peptide signals failed to relate to any particular mammal (data not shown). These findings suggest that this sample might not contain the critical component of Placenta Hominis. These results demonstrated that our method can also be used to examine Placenta Hominis, even in the presence of vegetal materials.

Placental extract containing nucleotide fragments can be identified by this method

Many injectable placental extract products in the market are used for antiaging and wound healing in clinics. Placental extract has also been introduced into cosmetics products based on the antiaging function. Earlier studies showed that polydeoxyribonucleotide (PDRN) is capable of stimulating vascular endothelial growth factor to trigger wound healing[26-28] and skin regeneration.[29] PDRN is a mixture of deoxyribonucleotide polymers with chain lengths ranging between 50 and 2000 bp and is very abundant in the placental extract.[17] Generally, placentas are available from livestock farms as by-products. However, the current process of producing placental extract means that proteins and nucleotides are hydrolyzed into small fragments, which makes it difficult to use them for species identification. Few products claim to contain DNA, but we were interested to see whether our method could be applied in this field. Eight injectable and cosmetics placental extract product samples were collected from different manufacturers made by different animal sources. Results showed that of the eight samples, the only sample that produced a DNA signal and generated a PCR-amplified product was an injectable placental extract product claimed to contain sheep extract. We could detect neither clear DNA signals by electrophoresis nor the PCR-amplified products from the rest of the samples. However, sequence results from the only sample containing DNA were unexpected. BLAST result showed 99% identity to salmon trout (*Oncorhynchus mykiss*) mtDNA with only a single base pair mismatch (16S: Seq ID-MF621750.1, identity: 99.59%, expect value: $2e^{-122}$; 12S: Seq ID-KP013084.1, identity: 97.64%, expect value: $5e^{-107}$) while the product claimed to be made from sheep placenta. Although sperm from salmon and trout is a good source of PDRN,[30,31] this should not be labeled as a placental extract and is a clear example of misbranded cosmetics, according to the FDA definition. With the unexpected result, we sent all these samples for proteomic analysis. However, due to protein hydrolysis and removal of larger residues during placental extract production, the peptide fragments from the samples were unable to make trustworthy species comparison (data not shown).

DISCUSSION

This method produced high copy numbers of mtDNA segments by PCR, increasing the rate of successful species identification. The mtDNA 12S and 16S sequences are composed of highly conserved regions and species-specific regions across different species, making it easier to separate different species using short sequences. The similarity between human and swine, cattle, and goat 16S fragments is 88%, 87%, and 87%

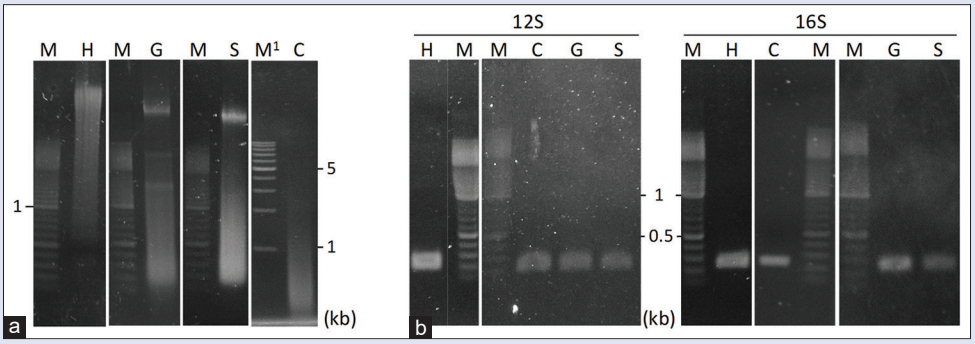

Figure 1: DNA isolation of fresh placenta and polymerase chain reaction amplification, (a) DNA extraction of fresh placenta. (b) Polymerase chain reaction amplification results of mitochondrial DNA 12S and 16S fragments. All samples were running in 0.8% agarose gels except cattle sample in (a) was running in 1.2% agarose gel. At column width. M: 100 bp ladder marker; M1: 1 kb ladder marker; H: Human; C: Cattle; G: Goat; S: Swine

while the similarity between 12S fragments of the same species above is 86%, 88%, and 85%, respectively [Figure 3]. Although being a powerful tool for protein identification, proteomics by mass spectrometry-driven sequence similarity searches[32] has its limitations. Especially with placental extract products, random digestion by hydrolysis, over-fragmentation during production treatments, and removal of larger peptide fragments increase the difficulty of applying this analysis. Compared with the use of protein identification, mtDNA identification is faster and cheaper and can be carried out by any molecular biology laboratory with standard equipment. This protocol does not need any commercial kit to isolate DNA from samples, and the DNA is amplified by the unified PCR condition with vertebrate universal primer sets. These processes require fewer laboratory resources, and DNA sequencing can be finished by self or sequencing companies. Compared with the existing COI-based sequence method, this method benefits from the following aspects: first, vertebrate universal primers are inactive with vegetal components or parasites, making sampling easier;[22] second, a single tube reaction instead of multiple tubes using different species-specific primers,[23] reducing the chance of contamination or handling error; and third, a kit-free sample preparation requires fewer resources. This method depends heavily on sequencing of the PCR products and is very sensitive to sample impurity/contamination because of specific amplification regions across species that could not be identified by size. Thus, positive/negative controls were introduced to prevent contamination. Theoretically, if there were multiple placental sources from different species in one sample, it can be distinguished from reading the sequencing peaks overlapped at species variation regions by directly sequencing the amplicons. In our study, no cross-species mixture in testing samples had been detected. This work is the first report identifying the vertebrate source of injectable placental extract products.

Figure 2: Morphology comparison of normal Placenta Hominis (left) and the one with no DNA trace and amplicon by vertebrate-specific primers (right). A paper-like membrane layer (red arrow) is underneath the yellowish vegetal coat. At column width

CONCLUSION

With the increase of placental products being adopted by the global market, it is essential to have better regulation rules and methods towards raw material inspection, effective ingredients analysis, detection of counterfeits and many more. In this work, we described a species identification method of placental products composed of sample preparation, mtDNA amplification by a unified PCR condition, and DNA sequencing. This method had been tested on the fresh placenta and Placenta Hominis, and the results were convincing and reproducible. For injectable and cosmetic products containing placental extract, only part of the samples containing nucleotides above a certain length can be detected with this method and one misbranded product was detected in this work. It is essential to identify the species of placental products because the limited legal source of human placenta is the main reason for counterfeit Placenta Hominis. Our results also showed the importance of precise regulation and standardized inspection methods for placenta-related products with massive market adoption by placental extract products, as a matter of public interest. Benefit from the nature of mtDNA, species identification can be carried with DNA fragments contained in products after different treatments through the production process while tissue/organ-specific identification could not be resolved by DNA-

12S

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              10         20         30         40         50         60         70         80         90
Human   CCCAAACTGG GATTAGATAC CCCACTATGC TTAGCCCTAA ACCTCAACAG TTAAATCAAC AAAACTGCTC GCCAGAACAC TACGAGCCAC
Swine   .......... .......... .......... C......... ...CA..T.. ...C..-... ......AT.. ......GT.. ...TC..A..
Goat    .......... .......... .......... ......... ..ACA..T.A ...C.GA... ....T.AT.. ......GT.. ...CG..A..
Cattle  .......... .......... .......... ......... ..ACAG.T.A ...C..A... ....T.AT.. ......GT.. ...T...A..

              100        110        120        130        140        150        160        170        180
Human   AGCTTAAAAC TCAAAGGACC TGGCGGTGCT TCATATCCCT CTAGAGGAGC CTGTTCTGTA ATCGATAAAC CCCGATCAAC CTCACCA-CC
Swine   T..C...... .........T .......... ...C....AC .......... .......A.. .......... ......AG.. ..T....A..
Goat    ...CCG.... .........T .......... .T...C..T. .......... .......A.. .......... ......A... .......AT.
Cattle  .......... .........T .......... .T......T. .......... .......A.. .......... ......A... .......ATT

              190        200        210        220
Human   TCTTGCT--- -CAGCCTATA TACCGCCATC TTCAGCAAAC
Swine   -.....CAAT T......... .......... ..........
Goat    -......AAT A...T..... .......... ..........
Cattle  -......AAT A...T..... .......... ..........
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16S

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              10         20         30         40         50         60         70         80         90
Human   GCCTGTTTAC CAAAAACATC ACCTCTAGCA TCACCAGTAT TAGAGGCACC GCCTGCCCAG TGAC-ACATG TTTAACGGCC GCGGTACCCT
Swine   .......... .......... .......... .T..T..... ........AT .......... ....-..CA. .......... ......TT..
Goat    .......... .......... .....C.... .TT....... .G.......T .......... ....T.A.C. ..A....... ......TT..
Cattle  .......... .......... .....C.... .TC....... .G......TT .......... ....-.AC.. .......... ......T...

              100        110        120        130        140        150        160        170        180
Human   AACCGTGCAA AGGTAGCATA ATCACTTGTT CCTTAAATAG GGACCTGTAT GAATGGCTCC ACGAGGGTTC AGCTGTCTCT TACTTTTAAC
Swine   .......... .......... .......... .TCC.....A ....T..... .......CA. .........T TA........ .....CC..T
Goat    .......... .......... ....T..... .TC......A ....T..... ...C...CA. .........T TA........ .....CC..T
Cattle  .......... .......... ....T..... .TC......A ....T..... .......CG. .........T TA........ .....CC..T

              190        200        210        220        230        240   245
Human   CAGTGAAATT GACCTGCCCG TGAAGAGGCG GGCATAACAC AGCAAGACGA GAAGACCCTA TGGAG
Swine   .......... A....T.... .......... ..A....A.A .AT....... .......... .....
Goat    .......... .....C.... .......... ..A..G.ATT .A........ .......... .....
Cattle  .......... .....T.... .......... ..A..GCACA .AT....... .......... .....
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Figure 3: Alignment of the polymerase chain reaction target sequences of 12S and 16S of four species. Using the human sequence as the reference, the identical and gap bases of other species are abbreviated by dot and dash, respectively. At full page width

based inspection. While this testing procedure can improve safety for the end consumers by preventing counterfeits from entering the market and providing better quality control for retail products, this method still needs to pair with other inspection tools for better identification when DNA trace was removed from many placental extract products.

Acknowledgements

The authors wish to thank Dr. Yeou-Guang Tsay and the Proteomics Research Center of National Yang-Ming University to complete this work.

Financial support and sponsorship

This study was funded by the Research Support Scheme of the Taiwan Food and Drug Administration, grant no. 102TFDA-A-521E.

Conflicts of interest

There are no conflicts of interest.

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