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Identification of Testudinis Carapax and Cervi Cornu in Kuei-Lu-Erh-Hsien-Chiao by Nested PCR and DNA Sequencing Methods

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ABSTRACT

Kuei-Lu-Erh-Hsien-Chiao (KLEHC) is widely used in Chinese medicine preparation for folk custom regimen, and is often sold through illegal suppliers. Testudinis Carapax and Cervi Cornu are the main raw materials of KLEHC. This study applied nested PCR and DNA sequencing method to identify the components of turtle and deer ingredients, which are the main constituents of KLEHC. The identification was based on the 12S rRNA gene sequences of mitochondria. This study modified the DNA extraction process to obtain the trace of DNA in KLEHC. With the sequences of the standards and GenBank database, two sets of primers were designed to specifically identify the turtle and deer components respectively. The results showed that all three samples from registered Chinese pharmaceutical manufacturers contained both turtle and deer components. Only seven in 26 samples from unlicensed suppliers contained both turtle and deer components, while 16 samples contained turtle or deer and three did not contain any. The results indicated that the proposed method could be employed to identify turtle and deer components in KLEHC.

Key words: Kuei-Lu-Erh-Hsien-Chiao, nested PCR, 12S rRNA

INTRODUCTION

Kuei-Lu-Erh-Hsien-Chiao (KLEHC) is a Chinese medicine preparation containing Testudinis Carapax, Cervi Cornu, Ginseng Radix and Lycii Fructus. Testudinis Carapax is the turtle shell and Cervi Cornu is the deer antler, both being the main raw materials of KLEHC. Ancient Chinese medical books indicated that it could be used to treat bone loss and weak eyesight. As a folk custom of Taiwan, it has been used as dietary supplement for regimen. Some KLEHC products were manufactured privately and sold illegally. Consumers are uncertain whether the preparation contains these components or it is beneficial to their health. Chinese medicinal preparations should be legally made by Chinese pharmaceutical manufacturers and sold by licensed dispensaries. Until now, the authorities have not established analytical procedures to check for the contents of turtle shell and deer antler in these preparations, and to inspect the preparations to determine whether they contain turtle shell drawn from endangered species.

KLEHC is produced by boiling the ingredients for a long time, so that the four raw materials in KLEHC lose their sharp edges. The chemical components of turtle shell and deer antler are not identifiable by chemical analysis. Thus, it is difficult to analyze their animal components with physical or chemical methods. DNA analysis by molecular biological methods may be feasible for identifying the components in KLEHC. However, the DNA of raw materials is destroyed after boiling, and DNA analysis may be difficult because DNA from turtle shell and deer antler could be cross-amplified in PCR. Therefore, it is necessary to develop a more specific and efficient PCR for DNA analysis.

DNA markers commonly used as animal species taxonomy are cytochrome b[1,2], 12S rRNA gene[3], and control region[4]. Based on the results of our previous study[5], the 12S rRNA gene in mitochondria was chosen in this study as the DNA marker. Turtle shell components in KLEHC could not be identified because PCR was cross-amplified with deer antler's DNA in the previous study. This study modified the DNA extraction process for the boiled samples, and applied nested PCR to discriminate between the two animal components. Therefore, deer and turtle species used in KLEHC preparation samples could be identified.

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MATERIALS AND METHODS

I. *Samples*

From 2005 to 2007, a total of 26 KLEHC samples were acquired by local health bureaus from illegal suppliers, such as folk medicine stores, unlicensed practitioners and others. Three authenticated KLEHC were purchased from Chinese medicine manufacturer in Taiwan.

II. *Primers*

We amplified the 12S rRNA gene fragment of mitochondria for turtle component by PCR with primer TuR2 (GCGGTGTGTGCGYRCCT, Y = C/T, R = A/G) and F (AGAAATGGGCTACATTTTCT), and then the PCR products were re-amplified by nested PCR with primer 2F1 (GCTACATTTTCTAYAY TAGAAAT, Y = C/T) and 2R1 (AGGACCGRCTTAAATTRGG, R = A/G). These primer pairs were designed according to 12S rRNA gene sequences of mitochondria of turtle species from GenBank database and the wobble theory. For deer component by PCR with primer CuF1 (GTCAAGGTGTAACCTATGGAAC) and R (GAGGGTGACGGGCG GTGTG), and then the PCR products were re-amplified by nested PCR with primer CuF2 (AGAAATGGGCTACATTTTCTAAT) and CuBtR1 (TGTGTGCGTGCTTCATG). These primer pairs were designed based on 12S rRNA gene sequences of mitochondria of deer species from GenBank database.

III. *DNA Extraction*

The DNA was extracted as described by Saghai-Maroof *et al*.[6] with modifications. First, we scrape ~100 mg glue from the KLEHC samples. The scraped glue of all samples was seperately placed in a 2 mL micro-centrifuge tube, digested in 1 mL lysis buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 1% N-lauroyl sarcosine sodium salt (sarcosyl), and 1 mg/mL proteinase K). The sample solution was incubated at 56°C for 1 hour, extracted with 1 mL phenol/chloroform/isoamylalcohol (25:24:1, v/v/v) mixture solution and centrifuged at 12,000 × g for 5 min. The aqueous solution was then transferred into another 2 mL micro-centrifuge tube. DNA in aqueous solution was precipitated by adding 0.7 mL isopropanol and 0.1 mL of 3 M sodium acetate, after which the solution was centrifuged at 12,000 × g for 5 min. Precipitated DNA was air-dried, and dissolved in 0.1 mL of sterile, distilled water. A PCR purification kit (QIAGEN GmbH, Germany) was used to purify the dissolved DNA, with the silica membrane to absorb DNA in the presence of high concentration of chaotropic salt in solutions. The purified DNA product could be reserved for further PCR analysis.

IV. *Polymerase Chain Reaction and 12S rRNA Gene Fragment Amplification*

All purified DNA were used as the PCR amplification template. Amplification was performed in 50 μL of solution by incubating 4 μL DNA with 5 μL 10 X *Taq* buffer, 0.5 μL of 25 μM PCR primer, 1 μL of 10 mM dNTP, 2 units of *Taq* polymerase, and sterilized distilled water. A negative-control (no template) reaction was included in each assay. For turtle component, mixtures containing the DNA extracted from samples were amplified by 30 cycles of PCR, each cycle consisting of a 30 sec denaturation step at 94°C, a 30 sec annealing step at 58°C, and a 30 sec extension step at 72°C. Two μL of the resultant PCR product was taken as a template for nested PCR. The temperature cycle of nested PCR was the same as prior PCR, except that the annealing temperature was 46°C. For deer component, mixtures containing the DNA extracted from samples were amplified by 30 cycles of PCR, each cycle consisting of a 30 sec denaturation step at 94°C, a 30 sec annealing step at 58°C, and a 30 sec extension step at 72°C. Two μL of the resultant PCR product was taken as a template for nested PCR. The temperature cycle of nested PCR was the same as prior PCR. Five μL of final PCR products were analyzed by electrophoresis in a 2% agarose gel. After electrophoresis in 0.5 X TBE buffer (Tris-Boric acid-EDTA) at 100 volts for 30 min, the gel was stained with ethidium bromide (0.5 mg/mL) before being photographed under ultraviolet (UV) illumination.

V. *Sequencing*

We submitted the final PCR products to sequence analysis by Mission Biotech (Taiwan) using the Applied Biosystems 3730 (ABI, USA). Sequence data were

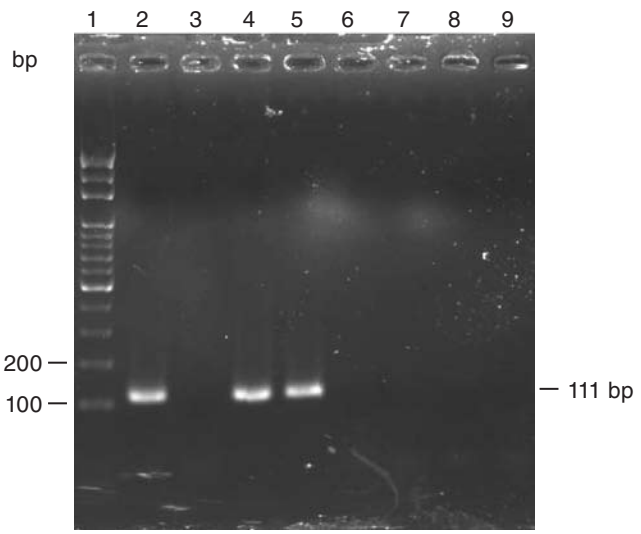

Figure 1. Electrophoresis of nested PCR product of turtle component in KLEHC. Lane 1: 100 bps ladder marker, Lane 2: authenticated KLEHC, Lane 3: sample 3, Lane 4: sample 10, Lane 5: sample 18, Lane 6: sample 22, Lane 7: sample 23, Lane 8: sample 24, Lane 9: blank (no template).

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compared with the GenBank database and the ITS sequence of the standard of deer antlers.

RESULTS AND DISCUSSION

In our previous study of KLEHC, turtle components were detected by PCR, and deer antler's DNA was cross-amplified, due to the primers of PCR with insufficient specificity for turtle's DNA. In this study, two specific primer sets and nested PCR were applied for specificity improvement. One set of primers from the previous study was modified, and another was a newly designed primer, based on the GenBank database. Total DNA of

Table 1. Results of turtle and deer ingredients identification from 26 KLEHC samples

No.	Date	Location	Turtle component	Deer component
1	2005.01	Taichung	ND[a]	ND
2	2005.03	Yunlin	ND	*Cervus unicolor*[b]
3	2005.07	Miaoli	ND	*Cervus elaphus*
4	2005.08	Yunlin	*Indotestudo elongate*	*Cervus nippon*
5	2005.11	Miaoli	*Siebenrockiella crassicollis*	*Cervus elaphus*
6	2005.11	Taitung	*Indotestudo elongate*	ND
7	2005.12	Taichung	*Heosemys grandis*	*Cervus elaphus*
8	2006.03	Taipei	*Melanochelys trijuga*	ND
9	2006.05	Miaoli	*Morenia ocellata*	*Cervus elaphus*
10	2006.05	Miaoli	*Indotestudo elongate*	*Cervus unicolor*
11	2006.08	Taipei	ND	*Cervus elaphus*
12	2006.09	Miaoli	*Morenia ocellata*	ND
13	2006.09	Yunlin	*Melanochelys trijuga*	ND
14	2006.11	Taichung	ND	*Cervus unicolor*
15	2006.11	Yunlin	ND	*Cervus elaphus*
16	2006.11	Yunlin	ND	ND
17	2006.11	Yunlin	ND	ND
18	2006.12	Penghu	*Siebenrockiella crassicollis*	*Cervus elaphus*
19	2007.01	Taichung	*Siebenrockiella crassicollis*	*Cervus elaphus*
20	2007.03	Tainan	*Heosemys grandis*	ND
21	2007.06	Pingtung	ND	*Cervus elaphus*
22	2007.07	Kaohsiung	ND	*Cervus unicolor*
23	2007.07	Kaohsiung	ND	*Cervus unicolor*
24	2007.07	Kaohsiung	ND	*Cervus unicolor*
25	2007.07	Kaohsiung	ND	*Cervus elaphus*
26	2007.08	Pingtung	ND	*Cervus unicolor*

[a] Not detected

[b] The result corresponds to GenBank access number: *Indotestudo elongate* - AF175338, *Siebenrockiella crassicollis* - AF043406, *Heosemys grandis* - AF043400, *Melanochelys trijuga* - AF043405, *Morenia ocellata* - AF043409, *Cervus unicolor* - AY184434.1, *Cervus elaphus* - AJ885206.1, *Cervus nippon* -AY184433.1.

samples were extracted and purified. A kit, originally used in PCR product purification, was used to eliminate the chemical components from unpurified DNA after the extraction process.

The primer sets for turtle component detection were examined by identifying the authenticated KLEHC to validate their specificity. After nested PCR, PCR products were produced from three authenticated KLEHC preparations. With the PCR products to be analyzed by DNA sequencing, turtle components of authenticated KLEHC were identified as one *Siebenrockiella crassicollis* and two *Melanochelys trijuga,* respectively. Then, the primer sets for turtle component detection were applied to 26 samples of KLEHC for nested PCR. The results of electrophoresis are shown in Figure 1. Among 26 samples of KLEHC, 14 contained no turtle components. The sequencing results indicated that five species of turtle were used in 12 samples, and showed no cross-amplified DNA of deer. The results are listed in Table 1. *Siebenrockiella crassicollis* and *Heosemys grandis* were found, which are listed in CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) Appendix II. Import of these species may be authorized by the granting of an export permit, or re-export certificate.

The certified deer antlers were taken to our Chinese medicine specimen room to be used as the standards. The antlers were from deer species included *Cervus elaphus*, *Cervus nippon, Cervus unicolor*, *Cervus axis*, and *Rangifer tarandus*. The antlers of these deer species were usually used for Chinese medicine preparation. The sequences acquired from the deer standards showed as Figure 2. DNA sequence data of deer's mitochondrial 12S rRNA gene were collected from the GenBank to check the sequences acquired from the deer standards. The sequences matched each other. All sequence data were used for primer design to detect deer component in KLEHC. Two set primers designed for deer component identification were applied on 26 samples of KLEHC for PCR and nested PCR. The results are shown in Figure 3. Nested PCR products amplified from 18 samples were sequenced. The sequences were compared with the standard results of deer components. The results are listed in Table 1. As seen in Table 1, 10 of the 26 samples contained *Cervus elaphus*, seven contained *Cervus unicolor*, and one contained *Cervus nippon*. Three authenticated KLEHC were tested, and the results showed that they all contained *Cervus elaphus.* According to the website of Deer Products Marketing Cooperative Association of Taiwan, *Cervus elaphus* has a higher yield of deer velvet in all breeds of deer, which may correspond to the results that more samples containing *Cervus elaphus* were identified.

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Cervus unicolor      AGAAATGGGCTACATTTTCTAATCTAAGAAAATCCAACACGAAAGTTATTATGAAATTAA
Cervus elaphus       ----------------------TC--------T--AAC------------------T--A
Cervus nippon        ----------------------TC--------T--AAC------------------C--A
Cervus axis          ----------------------TA--------T--ACT------------------T--G
Rangifer tarandus    ----------------------CT--------C--CCT------------------T--G

Cervus unicolor      TAACCAAAGGAGGATTTAGCAGTAAACTAAGAATAGAGTGCTTAGTTGAATTAGGCCATG
Cervus elaphus       ------------------------------------------------------C---------
Cervus nippon        ------------------------------------------------------T---------
Cervus axis          ------------------------------------------------------T---------
Rangifer tarandus    ------------------------------------------------------T---------

Cervus unicolor      AAGC
Cervus elaphus       ----
Cervus nippon        ----
Cervus axis          ----
Rangifer tarandus    ----
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Figure 2. The sequences acquired from the deer standards. A hyphen (-) indicates that the nucleotide is identical to the upper sequence.

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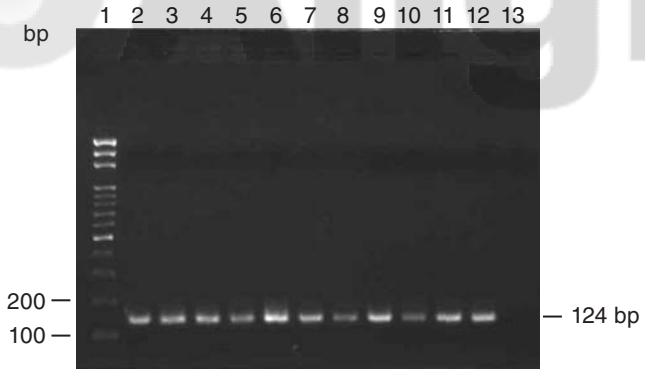

Figure 3. Electrophoresis of nested PCR product of deer component in KLEHC. Lane 1: 100 bps ladder marker, Lane 2: *Cervus elaphus*, Lane 3: *Cervus unicolor*, Lane 4: *Cervus axis*, Lane 5: *Rangifer tarandus*, Lane 6: authenticated KLEHC, Lane 7: Sample 3, Lane 8: Sample 10, Lane 9: Sample 18, Lane 10: Sample 22, Lane 11: Sample 23, Lane 12: Sample 24, Lane 13: blank (no template).

In Table 1, only seven of the 26 samples were confirmed to contain deer and turtle ingredients, while three samples detected neither deer nor turtle component, and were counterfeit. Other 16 samples contained either deer or turtle components. As mentioned above, three authenticated KLEHC purchased from legal Chinese pharmaceutical manufacturers contained deer and turtle constituents. The results indicated that illegal suppliers provided more counterfeit materials.

This study used specific primer sets combined with a nested PCR to solve the problem of cross-amplified DNA of turtle and deer, as discussed in our previous study. The modified DNA extraction process and nested PCR were useful to detect residual DNA in KLEHC. This method can be applied to identify the species of turtle and deer used in KLEHC, and differentiate authentic KLEHC from counterfeit. It is also helpful to reveal whether endangered species of turtle and deer are used to produce Chinese medicine.

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