Monitoring of Fasciola Species Contamination in Water Dropwort by COX1 Mitochondrial and ITS-2 rDNA Sequencing Analysis

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Abstract: Fascioliasis, a food-borne trematode zoonosis, is a disease primarily in cattle and sheep and occasionally in humans. Water dropwort (Oenanthe javanica), an aquatic perennial herb, is a common second intermediate host of Fasciola, and the fresh stems and leaves are widely used as a seasoning in the Korean diet. However, no information regarding Fasciola species contamination in water dropwort is available. Here, we collected 500 samples of water dropwort in 3 areas in Korea during February and March 2015, and the water dropwort contamination of Fasciola species was monitored by DNA sequencing analysis of the Fasciola hepatica and Fasciola gigantica specific mitochondrial cytochrome c oxidase subunit 1 (cox1) and nuclear ribosomal internal transcribed spacer 2 (ITS-2). Among the 500 samples assessed, the presence of F. hepatica cox1 and ITS-2 markers were detected in 2 samples, and F. hepatica contamination was confirmed by sequencing analysis. The nucleotide sequences of cox1 PCR products from the 2 F. hepatica-contaminated samples were 96.5% identical to the F. hepatica cox1 sequences in GenBank, whereas F. gigantica cox1 sequences were 46.8% similar with the sequence detected from the cox1 positive samples. However, F. gigantica cox1 and ITS-2 markers were not detected by PCR in the 500 samples of water dropwort. Collectively, in this survey of the water dropwort contamination with Fasciola species, very low prevalence of F. hepatica contamination was detected in the samples.

Key words: Fasciola species, water dropwort, cox1, ITS-2, DNA sequencing analysis

Fascioliasis is a zoonosis caused by Fasciola hepatica and Fasciola gigantica, 2 trematode species of the genus Fasciola, prevalent in cattle and emerging as a cause of disease in humans. Humans are infected mainly by ingesting raw water plants that are contaminated with the metacercariae [1]. Several reports have indicated that water plants such as watercress, rice, dandelion, Nasturtium, and Mentha spp. harbor Fasciola metacercariae [2].

Water dropwort (Oenanthe javanica) is a perennial herb with a distinctive aroma and is cultivated in marshy areas of Asia and Australia. The fresh stems and leaves are used as a salad or as a seasoning in soups and stews in Korea [3]. Water dropwort has also been used in Korea as a folk medicine for the treatment of jaundice, hypertension, fever, abdominal pain, leucorrhoea, mumps, and urinary difficulty [4]. In a biological hazard analysis of the water dropwort, it was reported that Escherichia coli was detected in samples of the herb collected from water dropwort fields [5]. However, there has been no information on Fasciola species contamination in water dropwort. Here, to obtain basic information regarding Fasciola species contamination in water dropwort in Korea, we collected a total of 500 samples from 3 areas, and evaluated Fasciola species contamination by mitochondrial cytochrome c oxidase subunit 1 (cox1) and nuclear ribosomal internal transcribed spacer 2 (ITS-2) DNA sequencing analysis.

Water dropwort samples were obtained between February and March 2015. A total of 500 samples were collected, and the lower parts of water dropwort was initially examined using a stereomicroscope (×10 magnification, Zeiss, Oberkochen, Germany). Next, we determined the presence of cox1 and ITS-2 genes of Fasciola species in each sample using PCR amplification. Briefly, the surface of the lower 20 cm of the water dropwort stem was peeled using a sterile scalpel, and genomic DNA was isolated using a G-DEX™ genomic DNA extraction
kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer’s instructions. Genomic DNA isolated from an adult *F. hepatica* worm (Prof. Sung-Jong Hong, Chung-Ang University, kindly provided) and adult *F. gigantica* worm (Prof. Keeseon S. Eom and Hyeong-Kyu Jeon, Chungbuk National University, kindly provided) were used as a positive control. The primers used for PCR amplification are listed in Table 1. The PCR mixture for the PCR amplification contained 5 μl genomic DNA, 3 μl each of forward and reverse primers, 4 μl dNTP, 5 μl 10× Ex Taq buffer, 0.25 μl Ex Taq polymerase, and 29.75 μl DDW. PCR assays were performed with an initial denaturation step of 94°C for 30 sec, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec, followed by 1 cycle at 72°C for 10 min and a final hold at 4°C. Amplifications were generated using a TaKaRa PCR Thermal Cycler (Takara Bio Inc., Otus, Japan). Agarose gel electrophoresis (1.5%) with ethidium bromide staining was used to visualize the ITS-2 PCR products. Furthermore, to identify the sequence of the PCR products from *Fasciola*-contaminated water dropwort, we purified the PCR products. Briefly, after electrophoretic separation, the *cox1* and ITS-2 PCR products were clearly delineated and sequenced directly by SolGent (Daejeon, Korea). The sequence of PCR products were compared with the complete *cox1* and ITS-2 sequences of *F. hepatica* obtained from GenBank (accession no. GU112476.1 and AJ272053.1, respectively) using Clone Manager software (Sci-Ed Software, Cary, North Carolina, USA). Also, the sequence of PCR products were compared with the

### Table 1. Primers used for detection of *Fasciola hepatica* and *F. gigantica* from water dropwort in Korea

| Target name   | Oligonucleotide sequence (5’-3’) | Product size (bp) | GenBank accession No. |
|---------------|----------------------------------|-------------------|-----------------------|
| *F. hepatica* COX1 | F: TTTGCGCTGGGTTTGAGTTA R: CCACACAAAGAGGATCCCATAT | 283 | GU112476.1 |
| *F. hepatica* ITS-2 | F: GTTATAAACATATCAAGAGCCTCCTCCAAA R: GAAGACAGACCAAGGGAAGGTA | 364 | AJ272053.1 |
| *F. gigantica* COX1 | F: GTCTTTGGGGTGGATTTTT R: GTCCAACCAACACCCATACC | 308 | AB983838.1 |
| *F. gigantica* ITS-2 | F: TATCACGAGCCAAAATG R: CGAGGGTAGGATCAGAAACA | 300 | EU260059.1 |

*COX1*, mitochondrial cytochrome *c* oxidase subunit 1; *ITS-2*, nuclear ribosomal internal transcribed spacer 2.

### Table 2. Results for the detection of the *cox1* and ITS-2 genes of *F. hepatica* or *F. gigantica* from water dropwort by PCR

| Areas | No. of samples | No. of PCR positive samples (%) |
|-------|----------------|--------------------------------|
|       |                | *F. hepatica* | *F. gigantica* |
| A     | 150            | 0 (0.0)       | 0 (0.0)       |
| B     | 200            | 1 (0.5)       | 0 (0.0)       |
| C     | 150            | 1 (0.67)      | 0 (0.0)       |
| Total | 500            | 2 (0.4)       | 0 (0.0)       |

**Fig. 1.** Agarose gel electrophoresis of PCR products containing the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) marker of *Fasciola hepatica*. M, 100 bp marker; lane 1, positive control (adult *F. hepatica* worm); lanes 2-7, *F. hepatica* negative samples of water dropwort; lane 8, No. 11 *F. hepatica* positive sample of water dropwort; lane 9; No. 18 *F. hepatica* positive sample of water dropwort.

**Fig. 2.** Agarose gel electrophoresis of PCR products containing the nuclear ribosomal internal transcribed spacer 2 (*ITS-2*) marker of *F. hepatica*. M, 100 bp marker; lane 1, positive control (adult *F. hepatica* worm); lanes 2-7, *F. hepatica* negative samples of water dropwort; lane 8, No. 11 *F. hepatica* positive sample of water dropwort, lane 9; No. 18 *F. hepatica* positive sample of water dropwort.
complete \textit{cox1} and ITS-2 sequences of \textit{F. gigantica} obtained from GenBank (accession no. ab983838.1 and EU260059.1, respectively).

We tried to detect the metacercariae of \textit{Fasciola} species from the surface of water dropwort using a stereomicroscope. However, metacercariae were not observed at the stack of water dropwort. Next, we performed PCR analysis on the 500 specimens of water dropwort to monitor \textit{Fasciola} species contamination. Among the 500 specimens collected from 3 areas, \textit{cox1} and ITS-2 bands of \textit{F. hepatica} were detected in 2 specimens (Table 2; Figs. 1, 2), which were exactly consistent with the PCR amplification of the positive control, adult \textit{F. hepatica} DNA (283 bp for \textit{cox1} and 346 bp for ITS-2). We also performed PCR analysis to evaluate \textit{F. gigantica} contamination of water dropwort using \textit{F. gigantica} \textit{cox1} and ITS-2 gene primers. However, the \textit{F. gigantica} \textit{cox1} and ITS-2 markers were not detected by PCR using 500 water dropwort (data not shown). To confirm whether the positive PCR products were real \textit{F. hepatica}, the complete DNA sequence of \textit{cox1} and ITS-2 PCR products were compared with those of GenBank. The nucleotide sequences of the \textit{cox1} PCR products from the 2 \textit{F. hepatica}-contaminated samples were 96.5% identical to the \textit{F. hepatica} \textit{cox1} sequences of GenBank (accession no. GU112476.1; Fig. 3), whereas \textit{F. gigantica} \textit{cox1} sequences were 46.8% similar with \textit{cox1} positive samples. The ITS-2 sequences of 2 PCR positive samples were 100% identical to those of GenBank (accession no. aj272053.1) and positive control sample (adult \textit{F. hepatica} worm); however, \textit{F. gigantica} ITS-2 sequences were 97.5% identical to those of ITS-2 positive PCR samples (Fig. 4). Thus, \textit{Fasciola} species PCR positive samples were confirmed to be \textit{F. hepatica}, and the overall prevalence of \textit{F. hepatica} infection in water dropwort was 0.4%, ranging from 0.0% to 0.67% depending on the collection area.

Fascioliasis in animals and humans is caused by \textit{F. hepatica} and \textit{F. gigantica}. It is difficult to accurately discriminate between 2 species because their size varies depending on the age of the fluke and species of the host [6-8]. PCR technology and DNA sequencing techniques facilitate species identification, clarification of strains, and genetic populations. Genes in the mitochondrial and nuclear DNA (the genes encoding ribo-
Fig. 4. *F. hepatica* ITS-2 nucleotide sequences of 2 positive samples obtained from PCR products compared with a GenBank sequence (accession no. AJ272053.1). Base homologies are indicated by a dot (·); base changes are shown in orange. Fh ITS-2 AJ27, *F. hepatica* ITS-2 GenBank sequence (accession no. AJ272053.1); No. 1, positive control (adult *F. hepatica* worm); No. 11, No. 11 *F. hepatica* positive sample of water dropwort; No. 18, No. 18 *F. hepatica* positive sample of water dropwort; Fg ITS-2 EU26, *F. gigantica* ITS-2 GenBank sequence (accession no. EU260059.1).

Somal RNAs) have been used as marker(s) in population genetics and phylogeny for fasciolid classification [6-8]. The prevalence of fascioliasis was greatly reduced in the 2000s in Korea. However, human cases of *F. hepatica* infection have been continuously reported [9,10]. Humans and cattle are most commonly infected by ingestion of water plants contaminated with encysted metacercariae. Water dropwort is one of the major sources of *F. hepatica* infection in Korea [2,9]. In this study, the overall prevalence of *F. hepatica* infection in water dropwort was 0.4%, which was much lower than that of snails in water dropwort fields in Korea [11]. Moreover, the prevalence in this study was lower than that in watercress in France (1.2-2.4% annually) [12]. Sources of *F. hepatica* contamination in agricultural products include soil, feces, irrigation water, inadequately composted manure, wild and domestic animals, dirty equipment, and human handling [13]. Differences in prevalence may be induced by various factors such as host distribution, locality, and environmental conditions. In this study, we used the repetitive DNA sequences of *cox1* and ITS-2 regions specific for *F. hepatica* or *F. gigantica* to identify the species of genus Fasciola because these genes were used efficiently to identify liver fluke species collected from various hosts and geographic regions [6-8]. From this study, *F. hepatica* cox1 and ITS-2 DNA were detected at 2 samples among 500 samples, but not *F. gigantica* contamination. These results were further confirmed by sequence analysis of positive PCR products in comparison to *cox1* and ITS-2 gene sequences of *F. hepatica* and *F. gigantica*.

Taken together, of 500 water dropwort samples, 2 water dropwort samples displayed the DNA bands of *F. hepatica* via PCR, and these findings were confirmed by sequencing analysis. This is the first study regarding parasitological examination of Fasciola species in water plants in Korea, suggesting that we need to improve the biosafety of aquatic plants during the pre- and postharvest periods.
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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

REFERENCES

1. Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol 2005; 35: 1255-1278.
2. Slifko TR, Smith HV, Rose JB. Emerging parasite zoonoses associated with water and food. Int J Parasitol 2000; 30: 1379-1393.
3. Seo WH, Baek HH. Identification of characteristic aroma active compounds from water dropwort (Oenanthe javanica DC). J Agri Food Chem 2005; 53: 6766-6700.
4. Yang SA, Jung YS, Lee SJ, Park SC, Kim MJ, Lee EJ, Byun HJ, Jhee KH, Lee SP. Hepatoprotective effects of fermented field water-dropwort (Oenanthe javanica) extract and its major constituents. Food Chem Toxicol 2014; 67: 154-160.
5. Kim YR, Lee KA, Choi IW, Lee YH, Kim SR, Kim WI, Ryu SH, Lee HS, Ryu JG, Kim HY. Investigation of microbial contamination in Oenanthe javanica at postharvest environments. J Food Hyg Safety 2014; 29: 268-277.
6. Itagaki T, Kikawa M, Terasaki K, Shibahara T, Fukuda K. Molecular characterization of parthenogenic Fasciola sp. in Korea on the basis of DNA sequences of ribosomal ITS1 and mitochondrial ND1 gene. J Vet Med Sci 2005; 67: 1115-1118.
7. Choe SE, Nguyen TT, Kang TG, Kweon CH, Kang SW. Genetic analysis of Fasciola isolates from cattle in Korea based on second internal transcribed spacer (ITS-2) sequence of nuclear ribosomal DNA. Parasitol Res 2011; 10: 833-839.
8. Wannasan A, Khositjarattanakool P, Chaiwong P, Piangjai S, Uparanukraw P, Morakote N. Identification of Fasciola species based on mitochondrial and nuclear DNA reveals the co-existence of intermediate Fasciola and Fasciola gigantica in Thailand. Exp Parasitol 2014; 146: 64-70.
9. Kim YH, Kang KJ, Kwon JH. Four cases of hepatic fascioliasis mimicking cholangiocarcinoma. Korean J Hepatol 2005; 11: 169-175.
10. Kang BK, Jung BK, Lee YS, Hwang IK, Lim H, Cho J, Hwang JH, Chai JY. A case of Fasciola hepatica infection mimicking cholangiocarcinoma and ITS-1 sequencing of the worm. Korean J Parasitol 2014; 52: 193-196.
11. Kim HY, Choi IW, Kim YR, Quan JH, Ismail HA, Cha GH, Hong SJ, Lee YH. Fasciola hepatica in snails collected from water-dropwort fields using PCR. Korean J Parasitol 2014; 52: 645-652.
12. Dreyfuss G, Vignoles P, Rondelaud D. Fasciola hepatica: epidemiological surveillance of natural watercress beds in central France. Parasitol Res 2005; 95: 278-282.
13. Berger CN, Sodha SV, Shaw RK, Griffin PM, Pink D, Hand P, Frankel G. Fresh fruit and vegetables as vehicles for the transmission of human pathogens. Environ Microbiol 2010; 12: 2385-2397.
