Species Identification of Medically Important Trematodes in Aquatic Food Samples Using PCR-RFLP Targeting 18S rRNA

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Abstract

The authors describe a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method targeting the 18S rRNA gene for the species detection of medically important trematode infecting fish and oysters, and suggest that this PCR-RFLP method based on a specific Tre-18 primer and the restriction enzymes, Acc1, Ava2, Msp1, and Hinf1, is useful for the detection of parasites in aquatic food.

Introduction

IT HAS BEEN ESTIMATED that more than 50 million people are infected with food-borne trematodes worldwide (Butt *et al.*, 2004). Many freshwater and brackish-water fish are involved with the transmission of medically important trematodes to people, and human intestinal trematode infections are endemic in many Asian countries (Nawa *et al.*, 2005; Chai *et al.*, 2007, 2009; Dung *et al.*, 2007; Shin *et al.*, 2008; Sohn *et al.*, 2009). In the Republic of Korea, infections with *Metagonimus yokogawai*, *Pygidiopsis summa*, *Heterophyopsis continua*, *Gymnophalloides seoi*, and *Clonorchis sinensis* examined in this study are relatively common (Chai *et al.*, 2009). These are viewed as medically important human parasites, which are transmitted in raw aquatic food in metacercaria form (Chai *et al.*, 2009). In particular, the *M. yokogawai* infection has been reported in Japan, Korea, China, Taiwan, and Russia (Chai *et al.*, 2009), and cases of *H. continua* and *P. summa* infections have been reported in Japan and Korea (Chai *et al.*, 2005, 2009). *C. sinensis* infection is highly prevalent in Korea, China, Taiwan, Vietnam, and Russia (Chai *et al.*, 2005, 2009). Recently, raw fish dishes have become popular worldwide, and the contamination of fish and oysters by parasites must therefore be properly monitored (Nawa *et al.*, 2005). Although microscopic observation provides an obvious method to detect parasites, polymerase chain reaction (PCR)–based methods have recently been used to detect metacercariae (larvae) in

seafood because of the objective nature of PCR analysis. Moreover, restriction fragment length polymorphism (RFLP) of PCR product targeting a specific gene is a good measure for the species discrimination of metacercariae isolated from fish.

A variable domain (V4 region) of the nuclear small subunit (18S) rRNA gene was found to be useful for phylogenetic studies because of the consistent differences in this gene between trematode species (Dzikowski *et al.*, 2004). Accordingly, the present study addresses the need to develop PCR-RFLP targeting the 18S rRNA gene for the detection and genetic identification of several types of trematode metacercariae infecting aquatic food.

Materials and Methods

Sweetfish (*Plecoglossus altivelis*), mullet (*Mugil cephalus*), striped shiner (*Pungtungia herzi*), and oysters (*Crassostrea gigas*) are collected from the Sum-jin River and brackish water areas in the Republic of Korea. Those fish and oysters are a second intermediate host of foodborne parasites such as *M. yokogawai*, *P. summa*, *H. continua*, *C. sinensis*, and *G. seoi*. To collect metacercariae, fish and oysters were ground using a motor mixer, digested with artificial gastric juice (0.5% pepsin [1:10,000, Sigma Chemical Co., St. Louis, MO] and 0.8% HCl) at 37° for 2–3 h, and metacercariae were recovered using microscopy.

Genomic DNA of metacercariae was purified using a DNeasy kit (Qiagen, Rodgau-Juegesheim, Germany) and

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FIG. 1. Species identification of medically important trematodes using polymerase chain reaction (PCR)–fragment length polymorphism. The genomic DNA of five types of trematode was amplified with Tre-18 primer (left panel). The size of undigested 18S rRNA PCR product is 733–739 bp. The band shown represents the PCR products of (1) *Clonorchis sinensis*, (2) *Gymnophalloides seoi*, (3) *Pygidioopsis summa*, (4) *Metagonimus yokogawai*, and (5) *Heterophyopsis continua*. The five types of trematode were digested with AccI, Ava 2, MspI, or HinfI (right panel).

specifically amplified with primers (Tre-18, which is our naming) (F): 5'-GATAACGGGTAACGGGGAAT-3', (R): 5'-AACCTCTGACTTTCGCTCCA-3' (2x PCR premix; Solgent Inc., Daejeon, Korea). The PCR condition is as follows: an initial denaturation at 95°C for 2 min, 35 amplification cycles (95°C for 20 s, 55°C for 30 s, and 72°C for 1 min), followed by one cycle at 72°C for 5 min. PCR products were sequenced using an ABI PRISM DNA analyzer (Applied Biosystems, CA). Gene alignments were performed using the Geneious Pro v5.0 program (Biomatters Ltd., Auckland, New Zealand). Subsequently, the species identification of trematodes was studied by RFLP analysis with AccI, Ava2, MspI, and HinfI. Products digested with restriction enzymes for 2 h at 37°C were run on 3% agarose gel and band patterns were visualized on a UV transilluminator and photographed using a Gel documentation system (UVP Upland, CA).

Results and Discussion

In the present study, specific primers (Tre-18) designed by the authors can amplify a partial DNA sequence (733–739 bp) of the 18S rRNA gene in metacercariae of five trematode species. The amplified partial coding sequences of five trematode species were provided to NCBI. Their GenBank accession numbers and product size are as follows: *G. seoi* (JQ955636-9, 733 bp), *C. sinensis* (JQ955640-2, 739 bp), *H. continua* (JQ955643-6, 738 bp), *P. summa* (JQ955647-955651, 739 bp), and *M. yokogawai* (JQ955652-6, 739 bp). After the digestion with each restriction enzyme, the DNA band pattern is shown in Figure 1. AccI and Ava2 digested the 18S rRNA of *G. seoi*. In contrast, MspI digested 18S rRNA of *C. sinensis* (271 bp and 240 bp), *P. summa* (352 bp and 263 bp), *M. yokogawai* (397 bp and 240 bp), and *H. continua* (397 bp and 239 bp), but not that of *G. seoi* (Figure 1). Although MspI digested the 18S rRNA of trematodes, it was difficult to differentiate *M. yokogawai* and *H. continua* because of their similar digested band patterns. HinfI digested the DNA of four trematodes, except *H. continua* (110 bp, 217 bp, and 382 bp for all four types

of trematode). These results suggest that AccI and Ava2 could be used to detect *G. seoi*, and that MspI could be used to detect the other trematodes. However, because *P. summa* and *H. continua* are often mixed infected in fish hosts such as *A. flavimanus* and *M. cephalus*, they can be also differentiated using HinfI, which selectively digests 18S rRNA of *P. summa*.

A recent increase in the numbers of infections caused by the consumption of undercooked aquatic food has been reported in Southeast and East Asia (Chai *et al.*, 2009). The significant problem in the detection of parasites in aquatic food is to confirm the mixed infection of parasites in certain foods. For example, the sweetfish, *Plecoglossus altivelis*, the dace, *Tribolodon* sp., and the perch, *Lateolabrax japonicus*, serve as second intermediate hosts for *M. yokogawai* and *H. continua* (Chai *et al.*, 2005, 2009). Brackish-water fish, such as the mullet, *M. cephalus*, the striped mullet, *Liza haematocheila*, the goby, *Acanthogobius flavimanus*, and the sweetfish, *P. altivelis* serve as second intermediate hosts for both *P. summa* and *H. continua* (Chai *et al.*, 2009; Cho *et al.*, 1985). Furthermore, human infection by *C. sinensis* is mediated by freshwater fish such as *Zacco platypus*, *Cyprinus carpio*, and *P. herzi* (Chai *et al.*, 2005, 2009). Accordingly, we suggest that our genetic test of metacercariae targeting 18S rRNA can be used to differentiate these parasites that infect fish and oysters. Although the test described cannot discriminate all trematode species, we suggest that PCR-RFLP targeting 18S rRNA can discriminate medically important trematodes endemic in East and South-east Asia.

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Disclosure Statement

No competing financial interests exist.

References

- Butt AA, Aldridge KE, Sanders CV. Infections related to the ingestion of seafood. Part II: Parasitic infections and food safety. *Lancet Infect Dis* 2004;4:294–300.
- Chai JY, Han ET, Guk SM, Shin EH, Sohn WM, Yong TS, Eom KS, Lee KH, Jeong HG, Ryang YS, Hoang EH, Phommasack B, Insisiengmay B, Lee SH, Rim HJ. High prevalence of liver and intestinal fluke infections among residents of Savannakhet province in Laos. *Korean J Parasitol* 2007;45:213–218.
- Chai JY, Murrell KD, Lymbery AJ. Fish-borne parasitic zoonoses: Status and issues. *Int J Parasitol* 2005;35:1233–1254.
- Chai JY, Shin EH, Lee SH, Rim HJ. Foodborne intestinal flukes in Southeast Asia. *Korean J Parasitol* 2009;47(suppl):S69–S102.
- Cho SY, Kim SI. *Plecoglossus altivelis* as a new fish intermediate host of *Heterophyopsis continua*. *Korean J Parasitol* 1985;23:173–174.
- Dung DT, De NV, Waikagul J, Dalsgaard A, Chai JY, Sohn WM, Murrell KD. Fishborne zoonotic intestinal trematodes, Vietnam. *Emerg Infect Dis* 2007;13:1828–1833.
- Dzikowski R, Levy MG, Poore MF, Flowers JR, Paperna I. Use of rDNA polymorphism for identification of Heterophyidae infecting freshwater fishes. *Dis Aquat Org* 2004;59:35–41.
- Nawa Y, Hatz C, Blum J. Sushi delights and parasites: The risk of fishborne and foodborne parasitic zoonoses in Asia. *Travel Med* 2005;41:1297–1303.
- Shin EH, Guk SM, Kim HJ, Lee SH, Chai JY. Trends in parasitic diseases in the Republic of Korea. *Trends Parasit* 2008;24:143–150.
- Sohn WM, Eom KS, Min DY, Rim HJ, Hoang EH, Yang Y, Li X. Fishborne trematode metacercariae in freshwater fish from Guangxi Zhuang autonomous region, China. *Korean J Parasitol* 2009;47:249–257.

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