



Differential Diagnosis of Human Sparganosis Using Multiplex PCR

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Abstract: Human sparganosis was diagnosed by morphological and genetic analyses in Korea. The complete mitochondrial genomes of *Spirometra erinaceiropaei* and *S. decipiens* isolated in Korea have been recorded. Present study was performed to provide information to diagnose the etiologic agent of sparganosis by multiplex PCR using mitochondrial genome sequences of *S. erinaceiropaei* and *S. decipiens*. In an effort to examine the differential diagnosis of spirometrid tapeworms, multiplex PCR assays were performed on plerocercoid larvae of *S. erinaceiropaei* and *S. decipiens*. The PCR products obtained using species-specific primers were positively detected in all PCR assays on mixture of *S. erinaceiropaei* and *S. decipiens* DNA. *S. erinaceiropaei*-specific bands (239 bp and 401 bp) were obtained from all PCR assays using a mixture of *S. erinaceiropaei*-specific primers (Se/Sd-1800F and Se-2018R; Se/Sd-7955F and Se-8356R) and *S. erinaceiropaei* template DNA. *S. decipiens*-specific bands (540 bp and 644 bp) were also detected in all PCR assays containing mixtures of *S. decipiens*-specific primers (Se/Sd-1800F and Sd-2317R; Se/Sd-7955F and Sd-8567R) and *S. decipiens* template DNA. Sequence analyses on these species-specific bands revealed 100% sequence identity with homologous regions of the mtDNA sequences of *S. erinaceiropaei* and *S. decipiens*. The multiplex PCR assay was useful for differential diagnosis of human sparganosis by detecting different sizes in species-specific bands.

Key words: *Spirometra erinaceiropaei*, *Spirometra decipiens*, differential diagnosis, multiplex PCR, human sparganosis

Human sparganosis is an infection caused by the plerocercoid larvae of the genus *Spirometra*, a kind of diphylobothriid tapeworms. These parasites require 2 different intermediate hosts: the fresh water copepods as the first intermediate host, and vertebrates such as amphibians, reptiles and mammals as the paratenic host. Humans become infected through ingestion of these intermediate hosts. *Spirometra erinaceiropaei* is the most well-known as the type species in the genus *Spirometra* (Cestoda: Diphylobothriidae) [1-4]. The plerocercoid larvae of spirometrid tapeworm (spargana) invade the brain, eyes, breast, spinal cord, and subcutaneous tissue of humans [5,6]. Sparganosis is a parasitic zoonosis that can cause serious clinical problems in human according to the invasive organs. More than 1,600 cases have been reported in the world. Most cases occurred in East and Southeast Asia, and sporadically in

South America, Europe and Africa [7]. The precise identification of these spirometrid tapeworms is important in efforts to control diseases that lead to human sparganosis.

Molecular identification has played an important role over the decades in studies pertaining to phylogenetics, parasite genetic variation and evolution. Mitochondrial DNA sequences have provided genetic markers for phylogenetic reconstruction, taxonomic identification, population genetics, and epidemiological investigations [8]. DNA sequencing of PCR products can be employed for molecular differential diagnosis and identification of *Spirometra* species using DNA sequence analysis of small (18S) and large (28S) subunit ribosomal RNA, ribosomal internal transcribed spacer 1, ribosomal internal transcribed 2, and mitochondrial genes [9-11]. Investigations of the phylogenetic relationships and genetic variations of *Spirometra* tapeworms have utilized mitochondrial genomes or genes such as cytochrome *c* oxidase subunit 1 (*cox1*) and 3 (*cox3*) and NADH dehydrogenase subunit 1 (*nad1*), 3 (*nad3*), and 4 (*nad4*) [12-17].

Recently, *Spirometra* species inducing human sparganosis in Korea have been identified by morphological and mitochon-

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drial *cox1* analysis [18], and the complete mitochondrial genomes of *S. erinaceiropaei* and *S. decipiens*, which cause human sparganosis, have been sequenced and analyzed [19]. These reports in Korea have included *Spirometra decipiens* as a possible causative agent for causing human sparganosis in addition to *S. erinaceiropaei*, the latter being a well-known single parasitic agent for human sparganosis. Therefore, a rapid and sensitive method is required for unambiguous species identification with differential diagnosis of human sparganosis. A reliable method for the identification of human infecting *Spirometra* tapeworms is necessary to assist with surveillance, disease control, and improve our understanding of the epidemiology of this foodborne zoonosis. In particular, questions remain concerning *S. erinaceiropaei* and its infection route (waterborne vs foodborne) in humans, and where in a recent paper on this parasite questions concerning the correct intermediate and final host animals in their life cycle were not addressed [18].

The aim of this study was to develop a high-resolution multiplex PCR assay using species-specific primers based on the mitochondrial sequences of *S. erinaceiropaei* and *S. decipiens* to distinguish between *S. erinaceiropaei* and *S. decipiens* that can be utilized for the differential diagnosis of human sparganosis from other cestodes.

A total of 25 parasites were used in this study, comprising 8 plerocercoids of *S. erinaceiropaei*, 7 plerocercoids of *S. decipiens* from infected humans in Korea, and negative control samples comprising *Taenia asiatica* (n = 1), *T. saginata* (n = 1), *T. pisiformis* (n = 1), *T. hydatigena* (n = 1), *T. crassiceps* (n = 1), *Hydatigera taeniaeformis* [18] (n = 1), *Echinococcus multilocularis* (n = 1), *Diphyllobothrium latum* (n = 1), and *D. nihonkaiense* (n = 1). For molecular characterization, all materials employed were identified by sequencing of the mitochondrial *cox1* gene.

Two sets of species-specific primers were designed to generate species-specific PCR products of different size based on the

nucleotide sequences of cytochrome *b* (*cob*), NADH dehydrogenase subunit 4L (*nad4L*), NADH dehydrogenase subunit 4 (*nad4*), cytochrome *c* oxidase subunit 1 (*cox1*), and large subunit ribosomal DNA (rDNA) from *S. erinaceiropaei* and *S. decipiens*. The forward primer was designed from a highly conserved region in *cob* gene, Se/Sd-1800F (5'-AGTTATTTTCGGTTGGTGCTGTAG-3'; nucleotides 1799 to 1822 from KJ599679 and nucleotides 1800 to 1823 from KJ599680). The 2 reverse primers were designed to amplify products of different length as follows: Se-2018R, specific for *S. erinaceiropaei* (5'-CCACAAACCAATAA-CAACTA-3'; nucleotides 2018 to 2039 from KJ599680); and Sd-2317R, specific for *S. decipiens* (5'-TCCTCCCCCACCACGACAAA-3'); nucleotides 2317 to 2337 from KJ599679.

The forward primer, Se/Sd-7955F (5'-ACGTGGTTTGTGTGGCTCATTTT-3'; nucleotides 7946 to 7969 from KJ599679 and nucleotides 7955 to 7978 from KJ599680), was from a highly conserved sequence common to both *Spirometra* tapeworms. The 2 species-specific reverse primers were designed from different positions of the *cox1* by mitochondrial by mitochondrial and rDNA regions as follows: Se-8356R, specific for *S. erinaceiropaei* (5'-ATGATAGGGTATAGGTGACCA-3'; nucleotides 8336 to 8356 from KJ588680), and Sd-8567R, specific for *S. decipiens* (5'-TTATTAACCTCCTAACCAACTTGATAC-3'; nucleotides 8567 to 8593 from KJ599679) (Table 1).

PCR assays were performed in 25 µl reaction mixtures comprising 100 ng of genomic DNA of each species, 10 pmol of each primer, 10× buffer, 10mM dNTP mix, 25 mM MgCl₂, and 2.5 unit *Taq* polymerase (High Fidelity PCR system, Roche, Mannheim, Germany). The multiplex PCR protocol consisted of 35 cycles of predenaturation (3 min at 94°C), denaturation (30 sec at 94°C), annealing (40 sec at 45°C), and extension (60 sec at 72°C), followed by 1 cycle of 5 min at 72°C.

PCR products on human sparganum DNA samples using species-specific primers and multiplex PCR assay are presented in Fig. 1. *S. erinaceiropaei*-specific bands (239 bp and 401

Table 1. Sequence and position of PCR primers used in present study

Primer	Primer sequence (5'-3')	Position in mitochondria gene
Se/Sd-1800F	TAT TTT CGG TTG GTG CTG TAG	<i>cob</i> (Sd, 1799-1822; Se, 1800-1823)
Se-2018R	CCA CAA ACC CAA TAA CAA ACT A'	<i>nad4L</i> (Se, 2018-2039)
Sd-2317R	TCC TCC CCC CAC ACG ACA AAA	<i>nad4</i> (Sd, 2317-2337)
Se/Sd-7955F	ACG TGG TTT GTG GTG GCT CAT TTT'	<i>cox1</i> (Sd, 7946-7969; Se, 7955-7978)
Se-8356R	ATG ATA GGG TAT AGG TGA CCA	rDNA (Se, 8336-8356)
Sd-8567R	TTA TTA ACT TCC TAA CCA ACT TGA TAC	rDNA (Sd, 8567-8593)

Se, *S. erinaceiropaei*; Sd, *S. decipiens*.

bp) were obtained from all PCR amplifications using a mixture of *S. erinaceiropaei*-specific primers (Se/Sd-1800F and Se-2018R; Se/Sd-7955F and Se-8356R) and *S. erinaceiropaei* DNA (Fig. 1, lanes 1 and 6). *S. decipiens*-specific bands (540 bp and 644 bp) were also detected in all PCR assays contain-

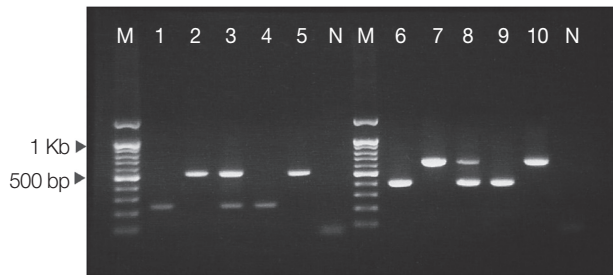


Fig. 1. Multiplex PCR performed with species-specific primer sets. Lanes 1 and 6, *S. erinaceiropaei* genomic DNA; lanes 2 and 7, *S. decipiens* genomic DNA; lanes 3 and 8, a mixture of *S. erinaceiropaei* and *S. decipiens* genomic DNAs; lanes 4 and 9, PCR reaction with *S. erinaceiropaei*-specific primers and a mixture of *S. erinaceiropaei* and *S. decipiens* genomic DNAs; lanes 5 and 10, PCR reaction with *S. decipiens*-specific primers and a mixture of *S. erinaceiropaei* and *S. decipiens* genomic DNAs; lane M, DNA size marker (100 bp ladder); N, negative control (no template).

ing mixtures of *S. decipiens*-specific primers (Se/Sd-1800F and Sd-2317R; Se/Sd-7955F and Sd-8567R) and *S. decipiens* template DNA (Fig. 1, lanes 2 and 7). Specificity of the PCR products was confirmed by sequencing. In an effort to differentially diagnose *Spirometra* plerocercoid infections using a single test, multiplex PCR assays were performed using a mixture of all primers and genomic DNA of *Spirometra* plerocercoid. Two sets of species-specific primers (Se/Sd-1800F and Se-2018R; Se/Sd-1800F and Sd-2317R) were combined in a single PCR assays using a mixture of *S. erinaceiropaei* and *S. decipiens* template DNA, which generated 239 bp and 540 bp band fragments (Fig. 1, lane 3). Another set of species-specific primers (Se/Sd-7955F and Se-8356R; Se/Sd-7955F and Sd-8567R) was combined in a single PCR assay using a mixture of *S. erinaceiropaei* and *S. decipiens* template DNA, which generated PCR products comprising 401 bp and 644 bp fragments (Fig. 1, lane 8). In an effort to examine the sensitivity of the species-specific primers, 2 sets of primers (Se/Sd-1800F and Se-2018R; Se/Sd-7955F and Se-8356R) were combined in a single PCR reaction containing *S. erinaceiropaei* DNA only (Fig. 1, lanes 4 and 9), and another 2 sets of primers (Se/Sd-1800F and Sd-2317R; Se/Sd-7955F and Sd-8567R) were used in a single PCR

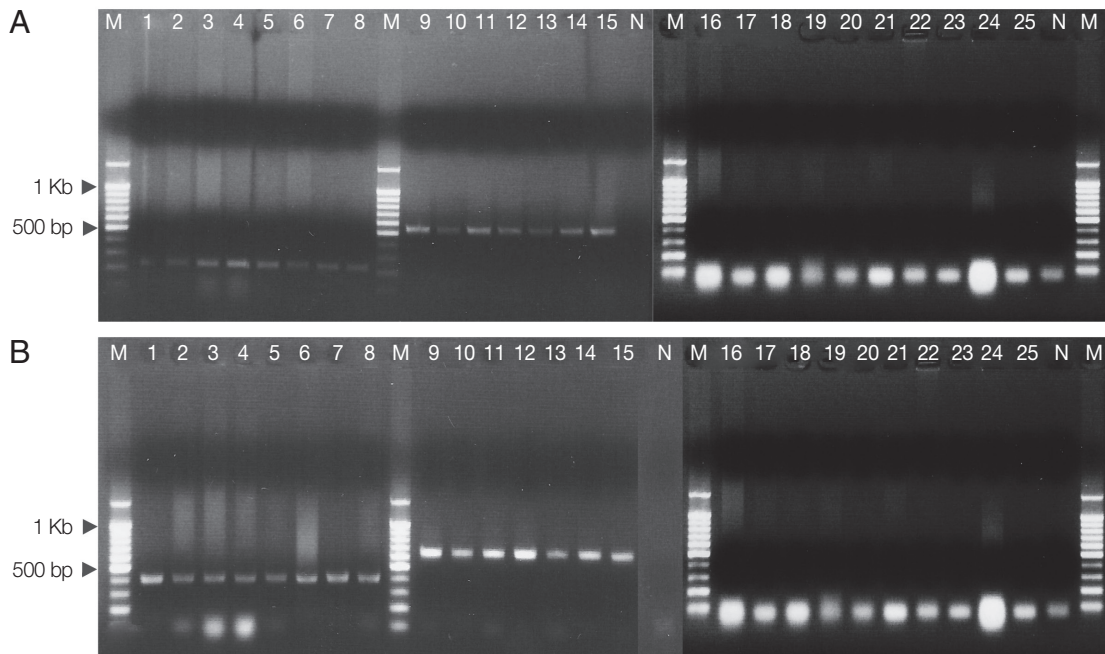


Fig. 2. Differential diagnosis of *Spirometra* tapeworms by multiplex PCR. (A) *S. erinaceiropaei* (lanes 1-8, 239 bp); *S. decipiens* (lanes 9-15, 540 bp). (B) *S. erinaceiropaei* (lanes 1-8, 401 bp); *S. decipiens* (lanes 9-15, 644 bp). Lane M, DNA size markers (100 bp ladder); *Taenia asiatica* (lane 16); *T. saginata* (lane 17); *T. solium* (lane 18); *Hydatigera taeniaeformis* (lane 19); *T. pisiformis* (lane 20); *T. hydatigena* (lane 21); *T. crassiceps* (lane 22); *Echinococcus multilocularis* (lane 23); *Diphyllbothrium latum* (lane 24); *D. nihonkaiense* (lane 25); N, Negative control (no template).

reaction containing *S. decipiens* template DNA only (Fig. 1, lanes 5 and 10).

To detect *Spirometra* plerocercoid infections, multiplex PCR assays were established utilizing DNA samples prepared from 15 cases of human sparganosis. Diagnostic bands, 239 bp and 401 bp, were produced by multiplex PCR of *S. erinaceiropaei* plerocercoid from human sparganosis (Fig. 2A, lanes 1-8; Fig. 2B, lanes 1-8), while 540 bp and 644 bp diagnostic bands were detected in the plerocercoid of *S. decipiens* from human sparganosis (Fig. 2A, lanes 9-15; Fig. 2B, lanes 9-15). No cross-reaction was detected with *S. erinaceiropaei*- or *S. decipiens*-specific primers when other genomic DNA was employed from human infecting tapeworms such as *Taenia*, *Echinococcus*, *Hydatigera*, and *Diphyllobothrium* template DNAs (Fig. 2A, B, lanes 16-25). No products were amplified from negative control samples by the multiplex PCR assay.

Since the first Korean sparganosis case described in 1954, human sparganosis has been reported in Korea in as many as 134 cases, involving cerebrospinal, subcutaneous, visceral, ocular, testicular, intramuscular, breast or multiple sparganosis. More than 1,600 cases of human sparganosis have been reported worldwide, with more than 80% of these being from China, Korea, Thailand, Japan, and the USA [7]. Although this genus comprises many species, a consensus on the genus *Spirometra* has not been achieved with respect to clear species classification. Many epidemiological questions pertaining to these parasites remain concerning species validity, geographical distribution, and transfer or final hosts. More recently, *S. erinaceiropaei* and *S. decipiens* have been identified from human sparganosis by morphological and genetic analysis in Korea [18]. These findings show that more than 2 *Spirometra* species are involved in human sparganosis in Korea, therefore, it is required to identify 2 *Spirometra* species of a causative agents for inducing human sparganosis. Differential identification of plerocercoids of the *Spirometra* species on the basis of morphological characters alone is problematic. Moreover, correct discrimination of plerocercoids of the *Spirometra* species obtained from various intermediate hosts using morphology has been considered nearly impractical. Molecular tools utilizing mitochondrial or nuclear genes have proven to be of diagnostic significance in the case of species of intermediate hosts that are related to human infections. Genetic markers could be useful in clarifying epidemiological, ecological, and geographical questions concerning *Spirometra* tapeworms. Differential diagnosis of the plerocercoids of *S. erinaceiropaei* and *S. decipiens*,

in addition to other cestodes, requires the establishment of molecular diagnostic techniques. A PCR-based molecular assay can be useful for the rapid and accurate differentiation of *S. erinaceiropaei* and *S. decipiens*, in addition to other cestode parasites.

Controversies remain concerning the species classification of tapeworms belonging to the genus *Spirometra*. The genus *Spirometra* has been studied intensively by many researchers since 1929 and a variety of spirometrid species have been identified [20-22]. Six *Spirometra* species were examined morphologically for species identification [20]. The *S. mansonoides* was identified from cats in the Syracuse region (USA) in 1937 [21]. Fourteen *Spirometra* species were recognized as being composed of 2 separate groups, the 'bresslauei' and 'okumurai' [22]. All species in the genus *Spirometra* were synonyms of *S. erinaceiropaei* [1]. Following extensive experimental studies of morphological variations, with particular emphasis on uterine coils, Iwata (1972) concluded that the morphological difference in uterine shape of the mature proglottids of *S. erinaceiropaei* was caused by differences in the developmental larval stages [3]. *S. erinaceiropaei*, *S. pretoriensis*, *S. theileri* and *S. mansonoides* were the only valid species designated by Kamo (1999) [2]. All *Spirometra* species found in Asian countries were considered synonymous with *S. erinaceiropaei* [1-3]. Approximately 38 species of the genus *Spirometra* have been reported from intestinal parasites of wild and domesticated carnivores [1-3,20,27]. Recent molecular data of these spirometrids suggests it includes many species of *Spirometra* from Asian, South American, and African isolates. Thirty-seven human sparganosis were reported from West African countries 34 specimens from South Sudan and 3 specimens from Ethiopia [23]. These human sparganosis was unclear and not be fully understood which *Spirometra* species related with human infections by mitochondrial *cox1* sequences analyzed. In Africa, the final hosts of *Spirometra* species were noted wild carnivores such as hyenas and lions. The parasite in the hyenas is known to be *S. pretoriensis* and that from the lions *S. theileri* [24-26]. The range of *Spirometra* species in Africa is unclear and not known relative role for wide host susceptibility in intermediate and paratenic hosts. The South America isolates of *Spirometra* spp. were reported from Brazilian isolates that revealed neither *S. erinaceiropaei* nor *S. decipiens* by mitochondrial *cox1* sequence analyzed. The partial *cox1* nucleotide divergence of Asian isolate identified as *S. decipiens* was differed from South America isolates (17.8-19.2%) while those of *S. erinacei-*

europaei (KJ599680) were differed 12.2-12.9% [27]. Those cases were differed from Asian isolates of *S. erinacei* and *S. decipiens* as well as those of African countries by analysis of mitochondrial DNA sequence data. These differences of taxonomic and phylogenetic results provoked epidemiological some questions concerning what is the geographical distribution of *Spirometra* spp. in worldwide? and which *Spirometra* species exists South America, Asian, and African countries? These inconsistencies suggest further studies are needed to better understand the taxonomic status of *Spirometra* spp.

In the present study, we developed a credible multiplex PCR assay for the differential identification of human infecting *Spirometra* species. The species-specific primers utilized in this multiplex PCR assay comprised 4 sets of species-specific primers that can generate PCR amplicons of different size from spirometrid parasites. The forward primers were derived from highly conserved sequences in the mitochondrial *cob* and *cox1* genes, and species-specific reverse primers were designed from highly unrelated sequences in *nad4L*, *nad4* and large subunit ribosomal DNA from both *Spirometra* tapeworms. This multiplex PCR assay is effective for differential diagnosis in the case of eggs, larva or proglottid, and has a detection sensitivity consisting of a minimum concentration of 50 eggs per gram from a fecal sample (data not shown). These fecal samples were stored in 0.85% saline or 70% ethanol at 20°C until use. Use of this multiplex PCR assay allows for differential identification of eggs, larva and adult worms, can assist in tracing the origin of the infective plerocercoids present in intermediate hosts, and can help to identify new intermediate hosts in addition to final host animals.

In conclusion, the results of the present study demonstrated that a multiplex PCR assay utilizing mtDNA sequences can be an effective and reliable molecular tool for the identification of *Spirometra* species causing human sparganosis. The multiplex PCR assay utilizing Se/Sd-1800F, Se-2018R, Se/Sd-7955F and Se-8356R primers for *S. erinacei*, and Se/Sd-1800F, Sd-2317R, Se/Sd-7955F and Sd-8567R primers for *S. decipiens* should be useful in the differential diagnosis, molecular characterization, and epidemiological survey of human sparganosis by *S. erinacei* and *S. decipiens*.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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