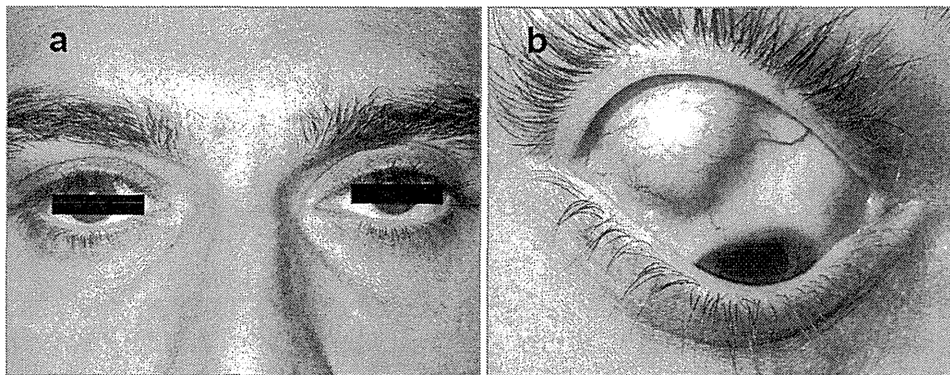

Fig. 1. Ophthalmologic findings. **a.** Right eye is slightly pseudoptotic; **b.** Mild lid edema and mild conjunctival hyperemia around the mass were seen

was superiotemporally located 5 mm away from the limbus. The mass also extended posteriorly through the orbital septum, near the superior rectus muscle. There was also a small amount of lid edema and conjunctival hyperemia around the mass. The patient's right eye was observed in a downward gaze (Fig. 1b). The patient had a corrected visual acuity of 20/20. In addition, the anterior and posterior segments as well as the intraocular pressure were all found to be normal in both eyes.

Orbital magnetic resonance imaging (MRI) was also used to properly discern the structure of the mass (Fig. 2). Both the signal and volume enlargement of the anteriosuperiolateral part of the right orbita were observed. Additionally, there was cystic formation between the sclera and the conjunctiva, with enlargement of the surrounding tissue. There were no other systemic findings.

A surgical excision of the mass was performed. During the surgery the semicystic mass was perforated and the parasite within was cut accidently. The parasite was then excised and

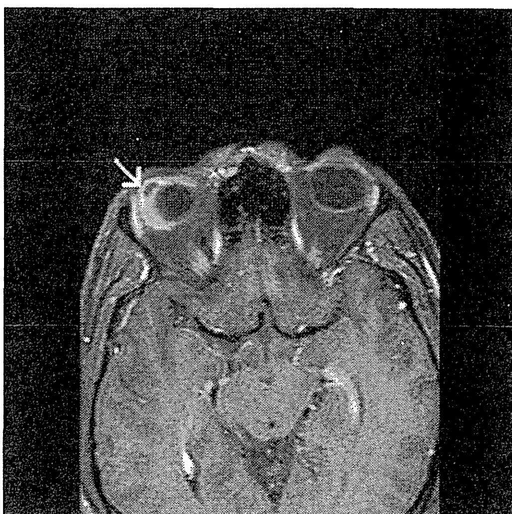

Fig. 2. Orbital MRI findings of the patient. White arrow showed the lesion. Note a cystic formation between sclera and conjunctiva with enlargement of right orbita

processed for histopathological examination. In order to identify the parasite to species at the DNA level, the formalin-fixed and paraffin-embedded (FFPE) specimen was sent to the Department of Parasitology at the National Institute of Infectious Diseases in Tokyo, Japan.

The patient returned 10 months later for a follow-up visit, and both the biomicroscopic and ophthalmoscopic examinations were normal. Furthermore, no parasite was found in the anterior chamber or in the vitreous cavity nor were there any other symptoms associated with the past infection.

DNA from the FFPE specimen was prepared using a DEXPAT® (Takara Bio, Japan), according to the manufacturer's instructions. For the molecular identification of the parasite, the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and NADH dehydrogenase subunit 5 (*nad5*) genes were amplified by polymerase chain reaction (PCR). The primers used were newly designated to be amplified short-size products (up to 250 bp) based on these genes of the filarial nematodes (*Onchocerca* and *Dirofilaria* spp.). The primers used were 5'-GCTTTGTCTTTTTGGTTTACTTTTG-3' and 5'-GTTGGG TGCTATTAATTTTATGG-3' for *cox1*, and 5'-CCTGTTA GTTGTTTGGTTCATAGTAG-3' and 5'-GGTTTTTGTTTT TTGGCTATTGGTAG-3' for *nad5*. PCR amplification was carried out in a total volume of 30 μL of reaction mixture with *ExTaq* DNA polymerase (TaKaRa Bio, Japan) and performed for 30 cycles of denaturation (98°C, 30 sec), annealing (54°C, 30 sec) and extension (72°C, 30 sec), plus one cycle of 72°C for 5 min. DNA sequencing was performed according to the method previously reported (Dang *et al.* 2010). Molecular identification was performed based on the genetic distance values and phylogenetic analysis of the cox1 and nad5 sequences.

Results

Morphological features of the parasite

Although the parasite was partially damaged (it had been accidentally cut during the surgical removal of the mass and its

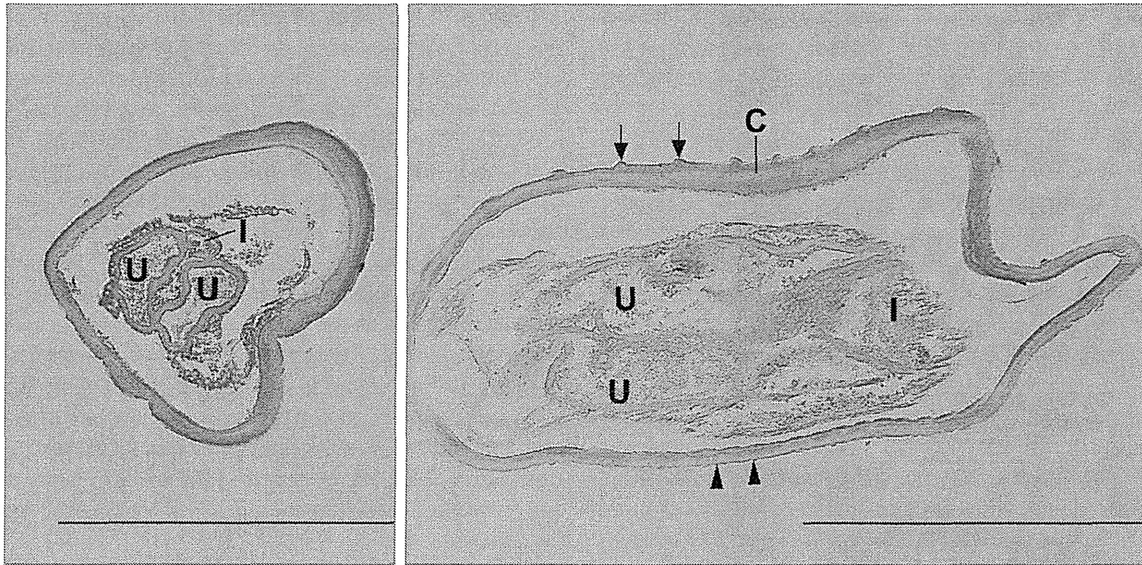

Fig. 3. Histopathological findings of the parasite. Arrows indicates prominent annular ridges on the external surface and transverse striae in the underlying layer are indicated by arrowheads. C, cuticle; U, uterus; I, intestine. Hematoxylin-eosin stain

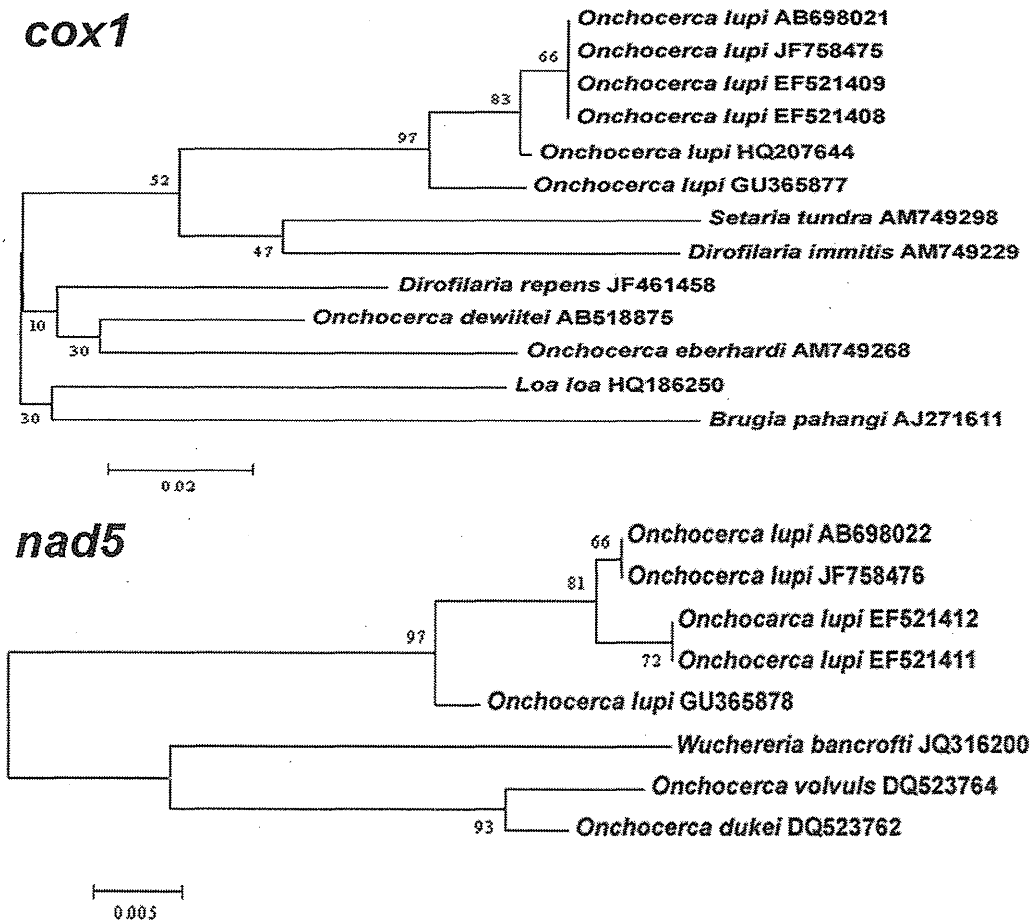

Fig. 4. Phylogenetic analysis of *Onchocerca* and other filarial nematodes inferred from the cox1 (above) and nad5 (below) genes by neighbor-joining method

internal organs had collapsed), some morphological features were able to be discerned (Fig. 3). The cross-section of the parasite showed a thick, multilayered cuticle (> 8μm) which had prominent annular ridges on the external surface (interval from 36.5–44.5 μm, arrows). Also, in the underlying layers of the cuticle transverse striae were observed (arrowheads in Fig. 3). The number of striae between consecutive annular ridges is useful in identifying *Onchocerca* species (Orihel *et al.* 1991 and Eberhard *et al.* 2000), and in this specimen there were two striae. In addition, reproductive organs with a thick wall were observed, strongly suggesting that this parasite was a female filarioid belonging to the genus *Onchocerca*.

Molecular analysis

Cox1 and *nad5* gene fragments were successfully amplified using PCR (data not shown). A comparison of the nucleotide sequences of *cox1* genes from our *Onchocerca* sp. specimen (AB698021) and *O. lupi* isolates from Europe found that the genetic distance values between the samples were low (d = 0.000~0.018), indicating typical intraspecific variation. A comparison of the *nad5* genes from our *Onchocerca* sp. (AB698022) and *O. lupi* isolates from Europe and the United States were also at values that correspond to intraspecific variation (d = 0.000~0.056). The filarial parasite was thus identified as *Onchocerca lupi*.

Discussion

The majority of infections caused by *O. lupi* were reported as ocular diseases in canines. It is thought that canines are the main hosts of *O. lupi* (Sréter and Széll 2008). It could therefore be the case that human infections are acquired accidentally, by some means.

The present study reports the third confirmed case of *O. lupi* in a human in Turkey, as confirmed by molecular analysis. Interestingly, there are no reports of *O. lupi* infections in dogs in Turkey. The patient reported in this study was from a different geographical area than the first two cases reported by Otranto *et al.* (2011, 2012). While the first two cases occurred in the northwest of Turkey, our patient came from southwest, about 550 km away from the previous two cases. The details of confirmed human cases caused by *Onchocerca lupi* are summarized in Table I. The other four ocular *Onchocerca* infections were diagnosed based on the morphology of the parasite.

Pampiglione *et al.* (2001) presented a subconjunctival infection caused by *Onchocerca* species, with the parasite being identified based on morphology (Pampiglione *et al.* 2001). Additional subconjunctival cases were verified by Otranto *et al.* using molecular analysis (Otranto 2011, 2012). In each of these subconjunctival infections, the patient had congestion, pain and inflammation. Our patient, however, had neither pain nor discomfort. Furthermore, although the cystic lesion was fixed to the sclera, there was no penetration. In the instance of Pampiglione *et al.*, for example, the parasitic mass penetrated both the sclera and the iris of the patient (Pampiglione *et al.* 2001). Sallo *et al.* (2005) reported a parasite in the vitreus cavity and Burr *et al.* (1998) reported one found in the cornea (Sallo *et al.* 2005 and Burr *et al.* 1998). Additionally, the first case presented by Azarova *et al.* (1965) was in the extraocular muscle tendon.

Although all of the previous cases reported in both humans and dogs were more symptomatic, our patient showed only mild inflammation. Therefore, *Onchocerca* infection should not be ruled out during the differential diagnosis of the subconjunctival and orbital cystic mass in instances where there is little to no inflammation.

Based on its scarcity in the literature, it would appear as though infection by *O. lupi* is rare in humans in general. However, filarial infections of the human eye are not uncommon. Often with these filarial infections, the individual parasite is not even identified to species level. In the present case, molecular analysis using a FFPE specimen was necessary to achieve a definitive identification of the parasite. All of this may suggest that other nematodes which infest the human eye may often be either under-identified (i.e. only to generic not specific level) or misidentified, thus obscuring the real frequency of *O. lupi* infections. It is therefore recommended that the parasite be identified to species level using molecular analysis whenever possible.

Furthermore, in order to better understand the life cycle of *O. lupi* and learn more about its threat to humans, additional research needs to be conducted to identify its reservoirs and vectors and its transmission patterns.

Acknowledgements. This work was supported in part by a Grant from Akdeniz University Scientific Projects Unit to HDI, and Grants-

Table I. Confirmed oncocercosis lupi in humans

Case#	Country	Age	Sex	Morphological identification	Molecular identification	Reference
1	Turkey	18	Female	*O. lupi*	*O. lupi*	Otranto *et al.* 2011
2	Turkey	26	Male	*O. lupi*	*O. lupi*	Otranto *et al.* 2012
3	Tunisia	8	NA†	*O. lupi*	ND‡	Otranto *et al.* 2012
4	Turkey	28	Male	*O. lupi*	*O. lupi*	The present case

† NA, not available
‡ ND, not determined

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CASE REPORT

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Sparganosis Presenting as Cauda Equina Syndrome with Molecular Identification of the Parasite in Tissue Sections

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Abstract: A 52-year-old woman presented with lower back pain, progressive symmetrical paraparesis with sensory impairment, and sphincter disturbance. Magnetic resonance imaging (MRI) of the whole spine revealed multiple intradural extramedullary serpiginous-mass lesions in the subarachnoid space continuously from the prepontine to the anterior part of the medulla oblongata levels, C7, T2-T8, and T12 vertebral levels distally until the end of the theca sac and filling-in the right S1 neural foramen. Sparganosis was diagnosed by demonstration of the sparganum in histopathological sections of surgically resected tissues and also by the presence of serum IgG antibodies by ELISA. DNA was extracted from unstained tissue sections, and a partial fragment of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene was amplified using a primer set specific for *Spirometra* spp. *cox1*. After sequencing of the PCR-amplicon and alignment of the nucleotide sequence data, the causative agent was identified as the larva of *Spirometra erinaceieuropaei*.

Key words: *Spirometra erinaceieuropaei*, sparganosis, cauda equina syndrome, molecular identification

INTRODUCTION

Human sparganosis is a zoonosis caused by plerocercoid larvae (=spargana) of pseudophyllidean (now classified as diphyllobothriidean [1,2]) tapeworms [3,4]. In Asia, *S. erinaceieuropaei* is responsible for this disease, whereas *Spirometra mansonoides* is important in the Americas. Sparganosis usually appears as slowly growing and migratory subcutaneous nodules. This parasite can be found anywhere in the body including the central nervous system [5,6]. In rare cases, a sparganum involves the spinal cord, usually at the thoracic to lumbar levels [7-15]. To our knowledge, only 3 cases have been reported as sparganosis with cauda equina syndrome [11,14,16]. None of these cases were confirmed by molecular diagnosis. Here, we describe a case of cauda equina syndrome with molecular evidence for identification of the causative agent and review the literature of sparganosis involving the spinal cord.

CASE RECORD

The patient was a 52-year-old female sugarcane farmer who lived in a rural community of Suphan Buri Province, central Thailand. She presented to our hospital complaining of lumbodorsal pain for the previous month, progressive symmetrical paraparesis with sensory impairment and radiculopathy for 2 weeks, and bowel/bladder dysfunction for 3 days. She had no history of ingesting inadequately cooked frogs, snakes, or birds. On examination, the patient had a normal body temperature. Skin nodules or organomegaly was not observed. Mental status and cranial nerve functions were within normal limits. Neurological examinations revealed decreased motor tone of both legs. Motor strength of the left lower extremity decreased from grade V (normal) to grade III in the hamstrings, iliopsoas, and quadriceps muscles; grade I-II in the ankle and toe plantar flexor muscles; and grade 0 in the ankle dorsiflexors muscles and the extensor hallucis longus muscle. For the

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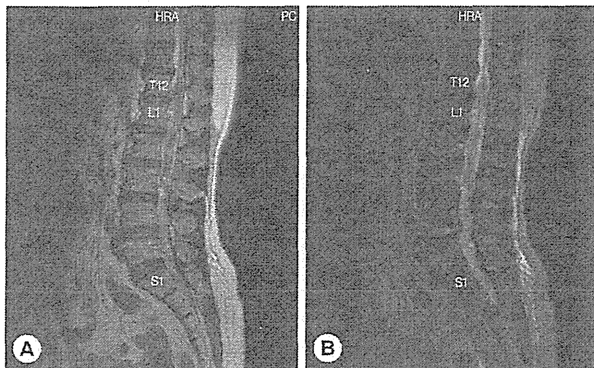

Fig. 1. Magnetic resonance of lumbosacral spine sagittal T2W image (A) and sagittal T2W with fat suppression image (B) shows abnormal lower lumbar spinal cord, conus medullaris, and cauda equina. Multiple heterogeneously serpigineous lesion filling within T12 to S1 of the thecal sac.

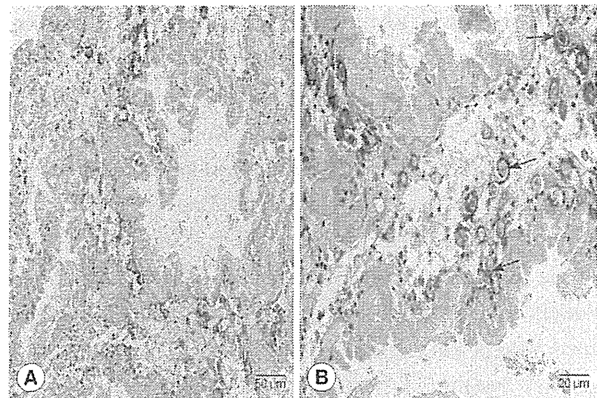

Fig. 2. Histopathological findings of the resected lesion. (A) Section of a degenerating cestode larva (molecular confirmed as *Spirometra erinaceieuropaei*) with homogenously eosinophilic and irregularly ridged tegument. Calcareous bodies and inflammatory cell infiltrations are seen throughout the parasite body. ×200. (B) Calcareous bodies (arrows) and mild inflammatory cell infiltrations including lymphocytes and a few eosinophils, plasma cells, and polymorphonuclear cells are seen in the body of the cestode larva. ×400.

right lower extremity, motor strength was decreased to grade III in all the muscles. Sensations were decreased below the L2 dermatome on both sides with peri-anal anesthesia, and the knee and ankle jerk were absent on both sides. Anal sphincter tone was reduced. A clinical diagnosis of cauda equina syndrome was made. Routine biochemical and hematological investigations were within normal limits except for an increase of absolute eosinophil count in the peripheral blood (0.46×10^9/L). MRI of the whole spine showed multiple intradural extramedullary serpiginous-mass lesions in the subarachnoid space continuously from the prepontine to the anterior part of the medulla oblongata levels, C7, T2-T8, and T12 vertebral levels distally until the end of the thecal sac, and filling-in of the right S1 neural foramen (Fig. 1). Attempts of spinal tapping to obtain a cerebrospinal fluid (CSF) sample were unsuccessful.

Laminectomy at L1 and L2 level was performed to obtain a tissue sample for histopathological examinations. During the operation, we noticed a matted mass involving nerve roots with remarkable inflammation and multiple cystic lesions containing pus; no CSF was seen due to obstruction by the mass. A small piece of tissue from the matted mass was removed and fixed in formalin and processed for paraffin embedding. The histological sections were stained with hematoxylin and eosin. Histological examinations demonstrated a degenerated solid section of a folded cestode larva having irregular folding tegument which was homogenously eosinophilic in color. Many calcareous corpuscles (Fig. 2B, arrow), which is a characteristic feature of a cestode larva, were seen. There were mild inflammatory cell infiltrations including eosinophils, lymphocytes, a few plasma cells, and neutrophils in the larval section (Fig. 2). The definite diagnosis of sparganosis could be made from the histopathological findings.

After the histopathological confirmation, serological tests for detection of specific antibodies in the serum was performed. Using ELISA, we detected a high titer of specific IgG antibodies against *S. erinaceieuropaei* sparganum partially purified antigen. The serum was negative for cysticercosis by ELISA and also negative for gnathostomiasis, paragonimiasis, fascioliasis, and angiostrongyliasis by immunoblotting.

For molecular identification of the causative parasite species, DNA was extracted from 10 μm unstained serial sections (cut from the formalin-fixed paraffin-embedded specimen) attached to glass slides using a DEXPAT kit (TaKaRa Bio Inc., Tokyo, Japan) as reported previously [17]. The resulting supernatants were used as the DNA template for PCR. Amplification of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene by PCR was performed in a 25 μl reaction mixture. A fragment of *cox1* gene was amplified using the primers Se658-F (5´-TTT GAT CCT TTG GGT GGT GG-3´) and Se1124-R (5´-ACC ACA AAC CAC GTG TCA TG-3´), which were designed from the *cox1* gene of *S. erinaceieuropaei* (GenBank accession no. AB369250). PCR was carried out using a GeneAmp PCR System 9700 (Applied Biosystems, Singapore). The reaction was carried out in a 25 μl volume containing 2.5 μl of 10x FastStart High Fidelity Reac-

tion buffer with 18 mM $MgCl_2$ (Roche, Mannheim, Germany), 200 μM of each deoxyribonucleotide triphosphate, 0.2 μM of each primer (Invitrogen, Carlsbad, California, USA), and 0.625 units of FastStart High Fidelity Enzyme Blend (Roche). The DNA template was initially denatured at 94˚C for 5 min. The amplification procedure consisted of 35 cycles at 95˚C for 30 sec (denaturation), 59˚C for 30 sec (annealing), and 72˚C for 45 sec (extension), with a final extension at 72˚C for 10 min. Amplified product was run on a 1% agarose gel; a 467 bp fragment was cut and sequenced using the MegaBACE™ 1000 DNA Analysis System (GE Healthcare, Piscataway, New Jersy, USA). The *cox1* gene sequence of *S. erinaceieuropaei* obtained from the patient was analyzed by BLAST-N search via NCBI.

The partial *cox1* sequence of *S. erinaceieuropaei* from the patient, which was deposited in the GenBank database (no. KC551943), was almost completely (97-99% identity) identical with those of *S. erinaceieuropaei* from various geographical localities. From these results, the parasite obtained from the patient was identified as *S. erinaceieuropaei*.

After laminectomy at L1 and L2 level, the patient received corticosteroid and praziquantel therapy, and her lumbosacral pain moderately improved. However, she persistently complained of a dull, uncomfortable sensation on both buttocks. At follow-up after 1 month, paraparesis with sensory impairment, urination, and defecation difficulties still persisted. This study protocol was approved by Siriraj Institutional Review Board Certificate of Approval (COA no. *Si* 189/2012).

DISCUSSION

Sparganosis is an uncommon disease in humans. It is caused by the larvae of the tapeworm genus *Spirometra*, whose definitive hosts are domestic and wild cats and dogs [11,14,16]. Human infections usually occur in the following 3 ways; drinking untreated water containing infected copepods; ingesting raw or inadequately cooked flesh of snakes, frogs, or birds infected with spargana; and applying the flesh of an infected frog as a poultice to a wound [18]. The route of infection of our patient remains uncertain. The disease usually involves the subcutaneous tissue or muscles of the chest, abdominal wall, or limbs. Central nervous system involvement is relatively rare, involvement of the spinal cord, in particular, is extremely rare [5,19]. In the literature [7-10,12-16], including the present study, 10 cases of spinal sparganosis have been reported. These were 6 males and 4 females whose ages ranged from 10 to 59 years (mean age; 38.9 years). The sparganum more commonly involved intradural (70%) than extradural [8,9,15] sites, usually at the thoracic level [7-10,12,13], followed by the cervical level [9]. Including the present case, there were only 3 cases of sparganosis involving the conus medullaris and cauda equina [13,14]. Our patient demonstrated long and multiple intradural extramedullary lesions extending from the prepontine to the anterior part of medulla oblongata levels, C7, T2-T8, and T12 vertebral levels distally until S1 level. Clinical manifestations included sensory disturbances (70%), weakness of the limbs (60%), pain (60%), and voiding difficulty (50%). The mean duration of symptoms before diagnosis was 9.4 months (range; 3 days to 3 years). All patients received surgical treatment; our patient received postoperative medical treatment. The prognosis was good or fair.

In nearly all of the spinal sparganosis patients, lesions were detected in the thoracic cord. Our patient was unique in that the lesions were at multiple levels from the prepontine down to the end of the thecal sac. The patient presented with severe lumbodorsal pain due to a mass compressing the adjacent spinal cord and resulting spinal cord edema. Pain in the nerve root may occur long before the signs of spinal cord compression have developed. In our patient, lumbodorsal pain developed before paraparesis and bowel/bladder involvement. The route of the entry of worms into the spinal cord remains unclear, but hematogenous spread seems likely.

It is hard to diagnose sparganosis clinically and to differentiate it from neoplastic and inflammatory disorders or other parasitic visceral larva migrans because it is rare and has nonspecific manifestations. We could not find any specific features for diagnosis in neuroradiological imaging of these cases. An immunological approach using ELISA is presently used for diagnostic purpose. ELISA for detecting anti-sparganum IgG antibody is highly sensitive (85.7-100%) and specific (95.7%) [20]. However, the ELISA technique can not identify the causative worm at the species level. PCR-based molecular techniques should be used for identification of the causative pathogens of infectious diseases. Recent development of PCR/sequencing technique for DNAs from formalin-fixed paraffin-embedded (FFPE) tissues kept for many years enabled us to perform the retrospective re-appraisal of individual cases and also epidemiological studies [21].

In conclusion, we report here a rare case of sparganosis presented as cauda equina syndrome. PCR-based identification of the causative agent using FFPE tissues led us to the accurate

and definite diagnosis.

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BRIEF COMMUNICATION

A Recombinant Matrix Metalloproteinase Protein from *Gnathostoma spinigerum* for Serodiagnosis of Neurognathostomiasis

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Abstract: Neurognathostomiasis is a severe form of human gnathostomiasis which can lead to disease and death. Diagnosis of neurognathostomiasis is made presumptively by using clinical manifestations. Immunoblotting, which recognizes antigenic components of molecular mass 21 kDa and 24 kDa in larval extracts of *Gnathostoma spinigerum* (Gs 21/24), has high sensitivity and specificity for diagnosis of neurognathostomiasis. However, only very small amounts of the Gs 21/24 antigens can be prepared from parasites harvested from natural or experimental animals. To overcome this problem, we recently produced a recombinant matrix metalloproteinase (rMMP) protein from *G. spinigerum*. In this study, we evaluated this rMMP alongside the Gs 21/24 antigens for serodiagnosis of human neurognathostomiasis. We studied sera from 40 patients from Srinagarind Hospital, Khon Kaen University, Thailand, with clinical criteria consistent with those of neurognathostomiasis, and sera from 30 healthy control adults from Thailand. All sera were tested for specific IgG antibodies against both *G. spinigerum* crude larval extract and rMMP protein using immunoblot analysis. The sensitivity and specificity for both antigenic preparations were all 100%. These results show that *G. spinigerum* rMMP protein can be used as an alternative diagnostic antigen, in place of larval extract, for serodiagnosis of neurognathostomiasis.

Key words: *Gnathostoma spinigerum*, neurognathostomiasis, recombinant matrix metalloproteinase, immunoblotting

Human gnathostomiasis is an important food-borne helminthic zoonosis caused by spirurid nematodes of the genus *Gnathostoma* and is endemic in Asia and the Americas [1-3]. Generally gnathostomiasis is not a life-threatening disease, fatalities being recorded only occasionally among neurognathostomiasis patients [2,4]. *Gnathostoma spinigerum* has been reported as the only causative agent for neurognathostomiasis in Southeast Asia, particularly Thailand, the only region where this condition exists [4]. Human infection occurs by consuming raw or inadequately cooked foods, e.g. freshwater fishes, frogs, and chickens, which harbor *Gnathostoma* advanced third stage larvae (AL3). The larvae migrate to the CNS to cause radiculomyelitis or radiculoencephalomyelitis. Subarachnoid hemorrhage (SAH) can occur, sometimes leading to death [5-7]. Definitive diagnosis of helminthic CNS infections is difficult. The chances of recovering worm specimens are extremely low [4]. The most reliable diagnostic tool is a serologic test, such as immunoblotting using *Gnathostoma* AL3 extract that is known to contain 2 antigenic peptides with approximate molecular masses of 21 kDa and 24 kDa [8]. However, only tiny quantities of the specific antigens can be obtained from AL3, and laboratory maintenance of the life-cycle to ensure supply is expensive and time consuming. Therefore, we evaluated the diagnostic use of a recombinant matrix metalloproteinase (rMMP) protein [9] for immunodiagnosis of neurognathostomiasis. Recombinant proteins can be produced in large quantities whenever required.

This study prospectively enrolled 40 patients who were sus-

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pected of having neurognathostomiasis at the Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand, between 2003 and 2012. A presumptive diagnosis of neurognathostomiasis was made using clinical criteria following a previous report [8]. The major criteria were eating raw food possibly contaminated with living *Gnathostoma* infective stage larvae from an endemic area, and acute-onset neurologic symptoms. Minor criteria were migratory swelling, radicular pain, blood eosinophilia >500 cells/mm^3, eosinophils in cerebrospinal fluid, and suggestive results by neuroimaging [7, 10,11]. A presumptive diagnosis of neurognathostomiasis was made when a person satisfied both of the major criteria and 1 of the minor criteria. All patients were positive for the 21 kDa and/or 24 kDa antigenic components of the *G. spinigerum* larval extract (Gs 21/24) but yielded negative serologic results for angiostrongyliasis [12], paragonimiasis [13], and fascioliasis [14] in immunoblotting, and also negative for cysticercosis [15] by ELISA. The negative control group included 30 samples from healthy adult volunteers who at the time of blood collection were found to be free from any intestinal parasite after stool examination using the formalin ethyl acetate concentration technique [16]. Informed consent was obtained from all human adult participants. The study protocol was approved by the Khon Kaen University Ethics Committee for Human Research (HE541293).

The rMMP protein was constructed from a cDNA encoding MMP protein of *G. spinigerum* larvae, cloned and expressed in *Escherichia coli* [9]. It had a molecular mass of approximately 102 kDa with the fusion-tagged protein. The *G. spinigerum* larval extract [17] and purified fusion-tagged rMMP protein [9] were separately characterized by SDS-PAGE followed by immunoblotting as previously described [17] with some modifications. The membranes blotted with *G. spinigerum* larval extract and rMMP were incubated in 1% skimmed milk in PBS, pH 7.5, containing 0.1% Tween-20 (PBST) for 30 min for blocking non-specific binding sites, washed, and then the membrane was cut into ~3 mm wide strips (9.8 µg protein/strip) for both antigens. Each strip was incubated with 1:100 diluted individual human serum sample (in 1% skimmed milk in PBS, pH 7.5) for 2 hr at room temperature. For immunoblotting against the rMMP protein, each serum sample was absorbed with *E. coli* lysate before use. The membranes were washed with 1% skimmed milk in PBST (5 times each) and incubated with goat anti-human IgG (H+L) HRP conjugate (Invitrogen) at a dilution of 1:4,000 in 1% skimmed milk in PBST, for 2 hr at room temperature. After 5 washes with 1% skimmed milk in

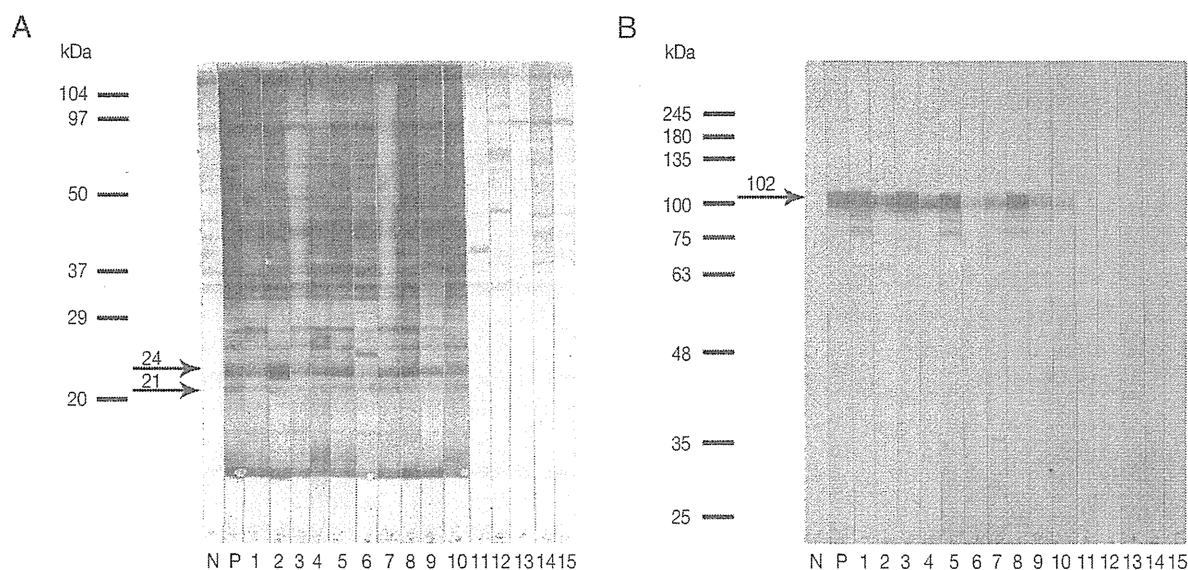

Fig. 1. Representative immunoblotting patterns (A, B) when human sera were tested against the *Gnathostoma spinigerum* larval extract containing 21 kDa and 24 kDa antigenic components (Gs 21/24) (A), and when the same sera were tested against the recombinant matrix metalloproteinase (rMMP) protein (B). Antigens were probed with the sera from pooled negative controls (N), pooled human gnathostomiasis patients (P), clinically confirmed neurognathostomiasis patients (1-10) and healthy controls (11-15). The arrows indicate the specific immunoreactive bands at approximately 21 kDa and 24 kDa (Gs 21/24) and at approximately 102 kDa (rMMP protein with the fusion-tagged protein).

PBST, the strips were developed with 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2 as substrate, and the reaction stopped with distilled water. The diagnostic parameters of sensitivity and specificity were calculated as previously described [18].

Immunoblot analysis employing the *G. spinigerum* larval extract of 21 kDa and 24 kDa antigenic components (Gs 21/24) (Fig. 1A) and the purified fusion-tagged rMMP protein (Fig. 1B) was evaluated using individual sera from healthy controls and from neurognathostomiasis patients. All serum samples from the neurognathostomiasis patients strongly reacted with both antigens. None of the 30 healthy control sera showed positive seroreactivity. The calculated diagnostic sensitivity and specificity were therefore 100% in each case.

Definitive diagnosis of neurognathostomiasis requires invasive procedures for demonstration of parasites [4]. The procedures need experienced personnel and laboratory support, and cannot be performed in general practice. Practically, diagnosis is made by clinical features, a history of eating parasite-contaminated foods, blood eosinophilia, eosinophils in cerebrospinal fluid, suggestive neuroimaging results, and serological findings especially via immunoblotting using the Gs 21/24 antigens [8]. Our results showed that the rMMP is as good as the Gs 21/24 antigens for serologic diagnosis of human neurognathostomiasis.

As neurognathostomiasis has been reported in Asia, i.e., Thailand, Laos, Japan, Mayanmar, and South Korea, as well as in European travelers returning from endemic areas [4], the mass production of *G. spinigerum* rMMP protein creates the potential for development of a serodiagnostic kit, i.e., immunochromatographic test. The method can be used not only for neurognathostomiasis but also for other forms of gnathostomiasis. The supportive diagnosis will help clinicians for prompt treatment of this harmful disease. Importantly, this diagnostic tool will be helpful for neurosurgeons considering management of intracranial hemorrhage patients without conventional invasive therapy.

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Molecular Detection of *Diphyllobothrium nihonkaiense* in Humans, China

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The cause of diphyllobothriosis in 5 persons in Harbin and Shanghai, China, during 2008–2011, initially attributed to the tapeworm *Diphyllobothrium latum*, was confirmed as *D. nihonkaiense* by using molecular analysis of expelled proglottids. The use of morphologic characteristics alone to identify this organism was inadequate and led to misidentification of the species.

Diphyllobothriosis is a fishborne cestodiasis caused by infection with adult tapeworms belonging to the genus *Diphyllobothrium* Cobbold, 1858 (*1–5*); the most frequent etiologic agents are *D. latum* and *D. nihonkaiense*. Humans are infected by ingesting raw or undercooked fish infected with larval plerocoercoids. Adult tapeworms can grow to »2 10 m in length in the human small intestine (*1–6*). Despite the large size of the tapeworms, clinical symptoms can be absent or mild and include mild abdominal pain, watery diarrhea, and abdominal discomfort (*3–7*). *D. latum* infection can also cause vitamin B_{12}-deficiency anemia (*5*).

Diphyllobothriosis caused by *D. nihonkaiense* has been extensively reported in Japan (*3,4*), but it has also occurred autochthonously in South Korea (*8*) and the Far Eastern Federal District of Russia (originally reported as *D. klebanovskii* infection [*9*]). Imported cases have been reported in Europe (*6*), North America (*10*), and New Zealand (*7*) in recent years.

In mainland China, 15 cases of diphyllobothriosis among humans have been reported since the first report in 1927 through 2012; the etiologic species was identified as *D. latum* by morphologic characteristics (*11–13*; Table) and molecular markers (*14,15*). No cases of diphyllobothriosis had been reported in large cities such as Beijing and Shanghai during 1951–2007 (*11*). However, we confirm 4 cases of *D. nihonkaiense* infection in humans in Shanghai, previously identified as *D. latum* infection, during 2008–2011, as well as 1 case in the moderately populous city of Harbin in Heilongjiang Province.

The Study

We examined 5 recent infections of humans with *Diphyllobothrium* spp. (Table, cases 12, 16–19) that occurred in China. Each case had been originally reported as a *D. latum* infection on the basis of morphologic identification only. Case 12 was reported in Harbin City, Heilongjiang Province, in 2009 (*15*). The 4 cases reported in Shanghai were diagnosed at the National Institute for Parasitic Diseases, Shanghai, on the basis of morphologic features of passed strobila. Case-patient 16 lived in Japan, but it was suggested that he acquired the tapeworm in Shanghai where he had frequently eaten raw salmon. Case-patient 17 was a 10-year-old girl from Japan. Whether she became infected in Shanghai or Japan was unclear because of lack of information. Case-patients 18 and 19 acquired the infection in Shanghai because they had never been abroad.

Because all patients in Shanghai had eaten raw salmon, we decided to re-examine how the causative *Diphyllobothrium* spp.were identified. *D. latum* infection is associated with consumption of freshwater fish such as perch (*Perca* spp.), not Pacific salmon (*Oncorhynchus keta, O. masou*) and Atlantic salmon (*Salmo salar*) in the Northern Hemisphere (*1–5*). To expand diagnostic parameters and clarify the point of misidentification, we re-identified *Diphyllobothrium* spp. by examining the tapeworms' morphologic features and using a molecular marker. In a sample from case-patient 12, only proglottids stained with acetic acid–carmine were available for testing by both methods (Figure 1, panel A). Proglottids obtained from 4 case-patients in Shanghai were preserved in either 10% formalin (case-patient 16) or 70% ethanol (case-patients 17–19) after collection (Table). Parts of the proglottids were embedded in paraffin, and sagittal sections were prepared for morphologic observation.

For molecular identification of the *Diphyllobothrium* spp., genomic DNA samples were extracted from specimens by using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). In specimens from case-patients 17–19, the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*, 1,566 bp) was amplified by PCR by using *Ex Taq* DNA polymerase (Takara Bio, Shiga, Japan) (*7*). In formalin-fixed samples of proglottids from case-patients 12

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Table. Cases of infection with *Diphyllobothrium* species in humans reported in mainland China, 1927–2012*

Case no.	Residence/place of eating fish, if different	Proglottids expelled	Suspected source of infection	Proglottid specimen fixative	Morphologic identification	Molecular identification	GenBank accession no. for *cox1* gene	Reference
1–4	Harbin/Heilongjiang	NA	NA	NA	*D. latum*	NT	NA	(*11*)
5	Beijing	NA	NA	NA	*D. latum*	NT	NA	(*11*)
6	Shanghai	NA	NA	NA	*D. latum*	NT	NA	(*11*)
7	Beijing (returned from United States)	NA	NA	NA	*D. latum*	NT	NA	(*11*)
8	Guangzhou (returned from Argentina)	NA	NA	NA	*D. latum*	NT	NA	(*11*)
9	Harbin/Heilongjiang	NA	Raw fish	NA	*D. latum*	NT	NA	(*11*)
10	Yanji/Jilin	NA	Raw fish	NA	*D. latum*	NT	NA	(*11*)
11	Fujian (lived in Yokohama, Japan, until 1996)	2003 Jan	*Plecoglossus altivelis*	NA	*D. latum*	NT	NA	(12)
12	Harbin/Heilongjiang	2009 Jan	Raw fish	10% formalin	*D. latum*	*D. nihonkaiense*	AB684625	(13) and this study
13	Heilongjiang	NA	Salmon	NA	*D. latum*	*D. latum*	NA	(*14*)
14	Jilin	NA	Salmon	NA	*D. latum*	*D. latum*	NA	(*14*)
15	Shanghai, 2008-2011/Japan, China	2011 Dec	Raw sea and freshwater fish	NA	*D. latum*	*D. latum*	NA	(*15*)
16	Shanghai/Japan (returned from Japan in June 2008; ate raw salmon in Shanghai)	2008 Oct	Raw salmon	10% formalin	*D. latum*	*D. nihonkaiense*	AB684624	This study
17	Shanghai	2011 Sep	Raw salmon	70% ethanol	*D. latum*	*D. nihonkaiense*	AB684621	This study
18	Shanghai. Ate raw salmon in April 2011	2011 Jun	Raw salmon	70% ethanol	*D. latum*	*D. nihonkaiense*	AB684622	This study
19	Shanghai. Ate raw salmon in 2011	2011 Jul	Raw salmon	70% ethanol	*D. latum*	*D. nihonkaiense*	AB684623	This study

*Identification of *Diphyllobothrium* spp. was performed by morphologic identification alone in cases 1–11; organisms in each case were identified as *D. latum*. Cases 12, 16–19 were assessed by morphologic and molecular identification; morphologic identification of all specimens was *D. latum*. Molecular identification varied from morphologic findings in 5 of 8 tested specimens.; NA, not available; NT, not tested.

and 16, DNA degradation caused by the fixative meant that only shorter *cox1* fragments (249 bp, corresponding to sites 880–1128 of *cox1*) could be amplified successfully by PCR by using KOD FX DNA polymerase (Toyobo, Osaka, Japan). DNA sequencing of amplicons was performed with a 3100-*Advant* Genetic Analyzer or 3730 xl DNA Analyzer (Life Technologies, Foster City, CA, USA). Phylogenetic analysis was performed by the maximum likelihood method (MEGA 5.05, http://megasoftware.net/mega.php) and Bayesian inference (MrBayes ver.3.1.2, http://mrbayes.sourceforge.net/). Clades were assessed by bootstrap resampling (1,000 replicates) and a posterior probability (10^6 generations) for the maximum likelihood and Bayesian inference trees, respectively. *Diphyllobothrium* spp. isolated from case-patients 12 and 16 were identified on the basis of sequence identity (%) by performing a BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis of a DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp).

Accurately identifyingthe *Diphyllobothrium* spp. isolated from case-patient 12 on the basis of morphologic features alone was difficult (Figure 1, panel A). In Figure 1, panels B–E show the sagittal sections of the proglottids from case-patients 16–19. The angle formed by the cirrus sac and the anterior–posterior axis of the proglottids was used as a criterion for differentiating *D. latum* from *D. nihonkaiense* (*1*), even though this criterion is not considered definitive: the angle is usually horizontal in *D. latum*, but oblique in *D. nihonkaiense*. Nonetheless, in this study, on the basis of morphologic criteria, tapeworms from case-patients 16, 17, and 19 were identified as *D. latum* (Figure 1, panels B, C, and E) and the tapeworm found in case-patient 18 was identified as *D. nihonkaiense* (Figure 1, panel D).

Phylogenetic trees based on the complete *cox1* nucleotide sequences showed the same topologies in maximum likelihood and Bayesian inference analyses, implying that the 3 isolates from persons in China (case-patients 17–19; GenBank [http://www.ncbi.nlm.nih.gov/genbank/] accession numbers AB684621–AB648623) are *D. nihonkaiense* (Figure 2). The 2 isolates (AB684625 and AB684624) from case-patients 12 and 16, respectively, were excluded from the analysis because they produced smaller PCR products, but they were identified as *D. nihonkaiense* on the basis of their 99%–100% sequence identity to *D. nihonkaiense*.

The 5 *Diphyllobothrium* spp. tapeworms examined in this study were previously identified as *D. latum* on the basis of morphologic characteristics, as were 3 of the 5 when we re-examined their morphologic characteristics. However, the 5 etiologic agents were confirmed as *D. nihonkaiense*

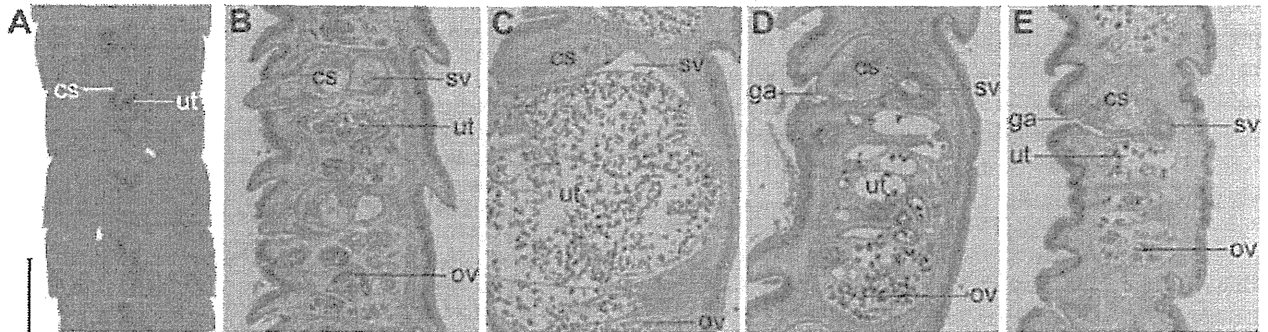

Figure 1. Diphyllobothriid samples examined in the present study, China, 2008–2012. A) Proglottids stained with acetic acid–carmine from case-patient 12. B–E) Sagittal sections of proglottids stained with hematoxylin-eosin from case-patients 16–19. cs, cirrus sac; ut, uterus; sv, seminal vesicle; ov, ovary; ga, genital atrium. Scale bar in panel A represents 2 mm; scale bars in panels B–E represent 500 µm.

by molecular analysis. This discrepancy in the identity of these agents may be attributed to the morphologic similarities between the species and the century-long confusion between the parasite *D. latum* and the parasite that caused human diphyllobothriosis associated with the consumption of Pacific salmon in Japan (*1–3*). Diphyllobothriosis caused by *D. nihonkaiense* has also been reported in South Korea (*10*) and in the Far Eastern Federal District of Russia (*11*) and is considered to be autochthonous and linked to the consumption of wild Pacific salmon in these regions. Therefore, some cases of diphyllobothriosis reported in mainland China were probably caused by infections with *D. nihonkaiense*; case-patient 12 (*15*) in this study is considered to have had such a case. However, a recent report stating that the causative species of 2 diphyllobothriosis cases in northeastern China was *D. latum* suggests that *D. latum* is also indigenous to mainland China (*8*).

Conclusions

We confirmed human diphyllobothriosis caused by *D. nihonkaiense* in mainland China by using a mitochondrial DNA marker. Reassessment of a case in Harbin revealed that some, if not all, of the autochthonous diphyllobothriosis cases were likely initially misdiagnosed as *D. latum* infection because of morphologic similarities between *D. nihonkaiense* and *D. latum* tapeworms. Consequently, molecular analysis is indispensable not only for avoiding diagnostic confusion among *Diphyllobothrium* spp., but also for facilitating the acquisition of reliable epidemiologic and epizootic information and improving clinical relevance and preventive controls for diphyllobothriosis.

Information on diphyllobothriosis and warnings of the potential risks associated with infection by its local species should be disseminated to food handlers, restaurant owners, physicians, and consumers. Because

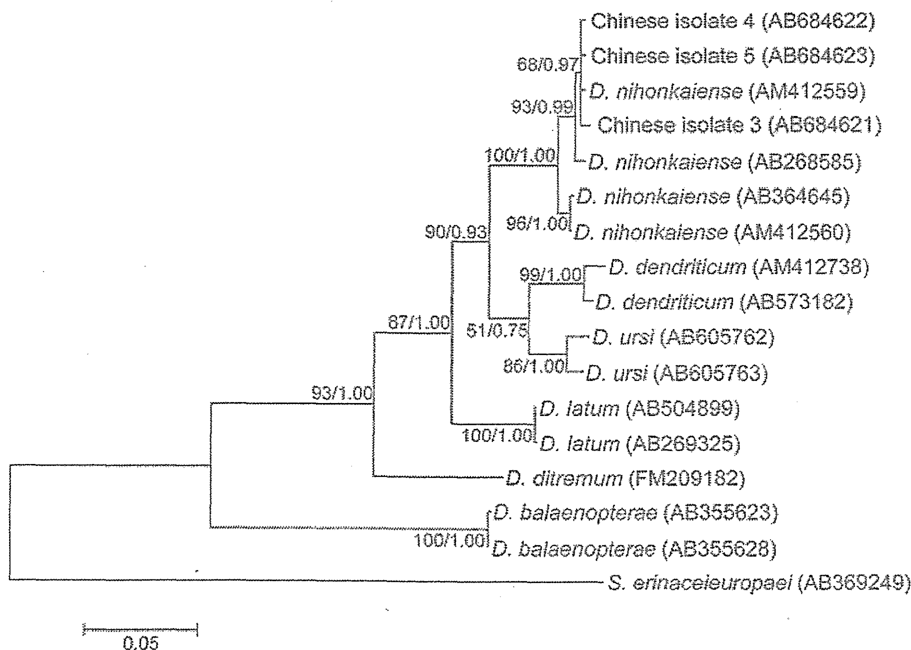

Figure 2. Phylogenetic tree constructed by using the maximum likelihood algorithm (Kimura's 2-parameter model) on the basis of the complete *cox1* sequences of isolates from *Diphyllobothrium* species found in persons in China and related *Diphyllobothrium* species. Numbers at nodes are bootstrap values (1,000 replicates) and posterior probabilities (10^6 generations) for maximum likelihood and Bayesian inference, respectively. *Spirometra erinaceieuropaei* was used as an outgroup. Scale bar indicates the number of base substitutions per site.

we cannot determine with certainty whether previous diphyllobothriosis cases in mainland China were caused by *D. latum* or *D. nihonkaiense*, identification of *Diphyllobothrium* spp. should be performed with care. In addition, studies on the distribution and sources of infection of *D. latum* and *D. nihonkaiense* on mainland China should be undertaken.

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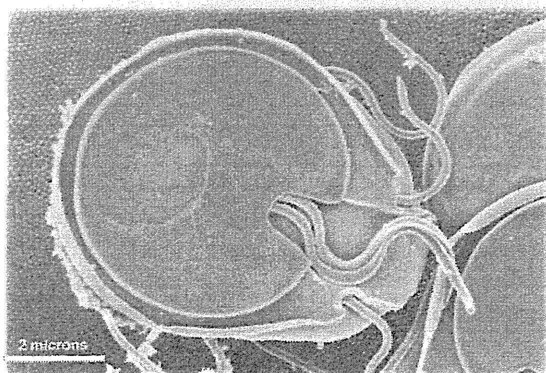

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アジア条虫（*Taenia asiatica*）感染症

はじめに

豚肉を感染源とする有鉤条虫（*Taenia solium*）や牛肉を感染源とする無鉤条虫（*Taenia saginata*）はよく知られた寄生虫であるが，これら2種類に加え，アジア条虫（*Taenia asiatica*）がヒトに寄生することは意外に知られていない．2012年6月頃から，これまでわが国にはいないと考えられていたアジア条虫によるヒトの感染事例が関東地方で相次ぎ，問題になっている[1)]．

アジア条虫とは

アジア条虫は東南アジアや韓国，台湾に分布し，形態学的には無鉤条虫に似た種であるが（図1-a），豚を中間宿主とする点では有鉤条虫に似る．アジア条虫の幼虫（＝囊虫，図1-b）は豚の肝臓に寄生するので，ヒトは豚の肝臓を生食することによって感染する．2〜3カ月の潜伏期を経て，ヒトの小腸で成虫になり，片節が排出される．おもな症状は軽度の腹痛や下痢，片節排出による不快感などである．

国内における疫学的背景

2010年6月から2012年10月までに，関東地方に限局してアジア条虫感染者が25名確認されている．多くは群馬，栃木，埼玉県在住者で，海外渡航歴のない日本人であった．30〜50歳代が多く，男女比は1.5：1であった．感染者は自宅や焼肉店などで豚レバー刺しを喫食していた．日本にはいないはずのアジア条虫がなぜ関東地方に限局して発生しているのか，その原因は調査中でいまだ明らかではない．しかし，養豚施設内に外国人アジア条虫感染者が立ち入り，感染者から排出された片節を豚が摂取して感染し，その感染豚の肝臓が流通した可能性が高い．

DNA鑑別法

アジア条虫と近縁種との鑑別法として，ミトコンドリアゲノムに存在するcytochrome *c* oxidase subunit 1遺伝子（*cox1*，全長1,620 bp）をpolymerase chain reaction（PCR）によって増幅し，その増幅産物が特定の制限酵素で切断されるか否

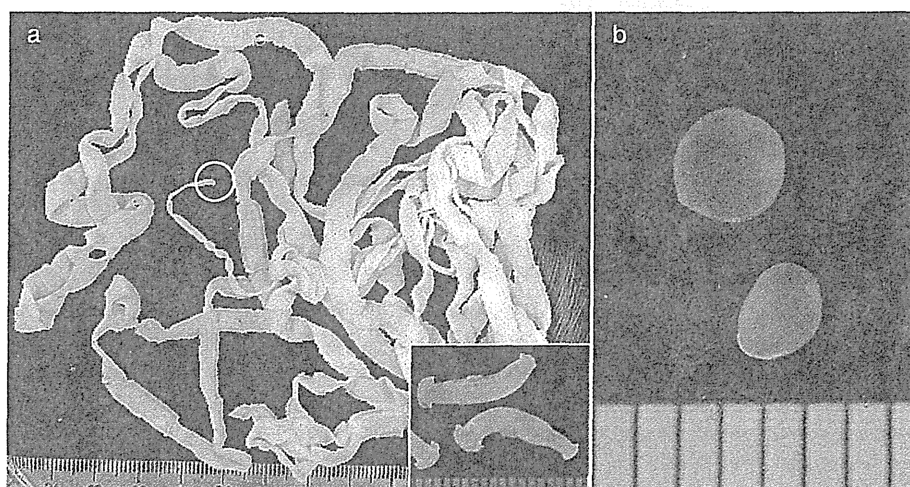

図1　アジア条虫

a：駆虫された成虫（体長約3 m）．駆虫しないと，片節がちぎれて肛門から這い出てくる（右下）．円内は頭節．

b：ヒトへの感染型である幼虫（囊虫）は，透明感のある白色球形（径2〜3 mm）．

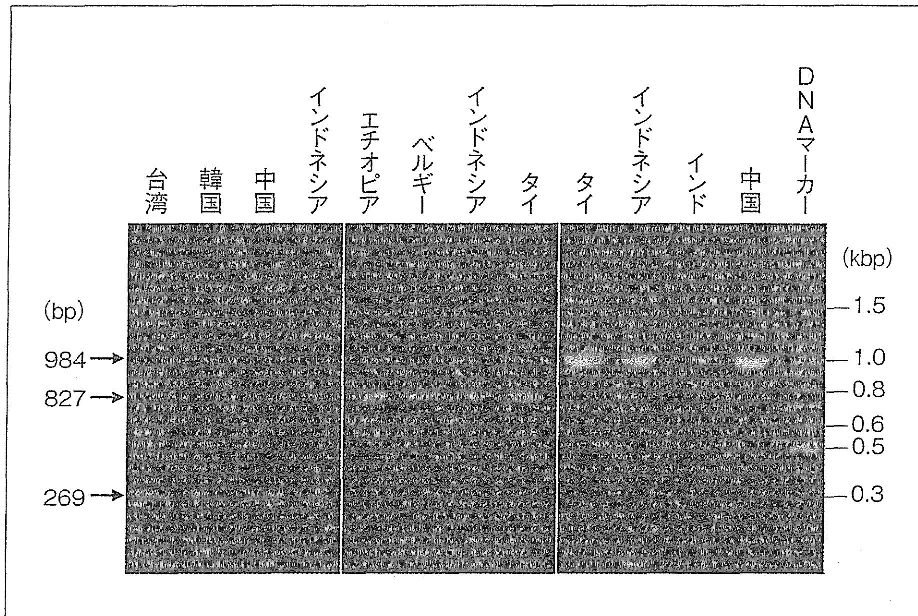

図 2 *cox1* 遺伝子を標的にした multiplex PCR による DNA 鑑別例
(Yamasaki, H., et al.; 2004[3] を改変)
国名は，鑑別診断に用いた虫体が採取された国を示す．
アジア条虫であれば 269 bp，無鉤条虫では 827 bp，有鉤条虫であれば 984 bp の産物が増幅される．

かで鑑別する方法や，増幅産物の大きさで鑑別する multiplex PCR（図 2）が確立されている[2,3]．また，*cox1* 遺伝子の塩基配列解析により 723 番目の塩基がグアニン（G）であればアジア条虫，アデニン（A）であれば無鉤条虫，シトシン（C）またはチミン（T）であれば有鉤条虫と同定できる．さらに，*ef1-α* 遺伝子や *elp* 遺伝子解析でも，アジア条虫と無鉤条虫は鑑別できる[4]．

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講座

食品による寄生動物感染症 8

蠕虫感染症 （3）条虫

山﨑　浩

1．はじめに

条虫は扁形動物門，条虫綱に属する大きな生物群で，成虫は真田紐の模様に似ていることからサナダムシとも呼ばれる。ヒトに健康被害を及ぼす種類は裂頭条虫目（Diphyllobothriidea）と円葉目（Cyclophyllidea）に属し，前者には裂頭条虫や複殖門条虫，後者には有鉤条虫や無鉤条虫などがそれぞれ含まれる。従来，用いられていた擬葉目（Pseudophyllidea）は，現在，裂頭条虫目と吸頭条虫目（Bothriocephalidea）の２目に分割されたために，以下に述べる裂頭条虫類は裂頭条虫目に属することになる[1]。

わが国では，食品媒介性条虫症は古来より発生していたが，現在でも日本人の食文化や食習慣の多様化によって発生が後を絶たない。また，邦人の渡航者数の増加に伴い海外で感染し，帰国後発症する例，あるいは，来日外国人の症例など，いわゆる輸入寄生虫症として増加傾向が見られる。本稿では，紙面の都合上，わが国で公衆衛生上，ならび食品衛生上問題になる魚介類と食肉の生食に起因する主な条虫症について，最新の知見を含めて概説する。

2．日本海裂頭条虫（*Diphyllobothrium nihonkaiense*）と広節裂頭条虫（*Diphyllobothrium latum*）

1）分類・形態

裂頭条虫属は38種で構成され，少なくとも11種がヒトに寄生する[2]。広節裂頭条虫（*Diphyllobothrium latum*）は古くからよく知られた種であるが，わが国で問題になる種は日本海裂頭条虫（*Diphyllobothrium nihonkaiense*）と，稀に，太平洋裂頭条虫など数種の海洋性裂頭条虫である。わが国における裂頭条虫症は1889年に広節裂頭条虫症として報告されたのが最初である。以来，約100年にわたり1,800例近い症例が報告されてきた[3]。しかし，日本の広節裂頭条虫はフィンランド産の広節裂頭条虫とは生活史が異なり，また内部形態

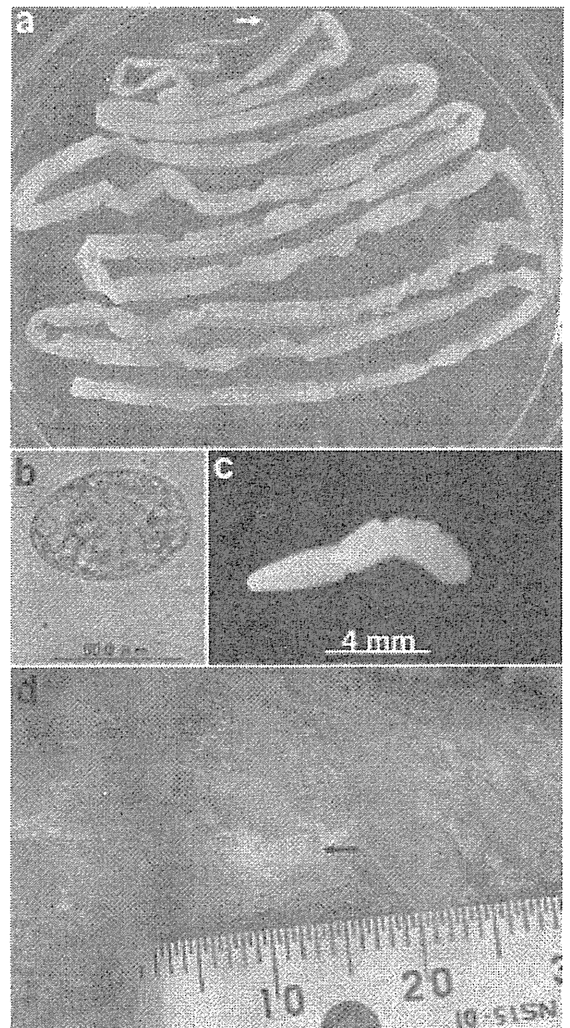

図１．日本海裂頭条虫
a, ヒト患者から駆虫された成虫（矢印は頭節）。片節中央に虫卵が充満した子宮が観察される；b, 虫卵；c, シロザケから採取されたプレロセルコイド（京都府立医大・山田 稔先生提供）；d, シロザケの筋肉内に寄生するプレロセルコイド（旭川医科大・中尾 稔先生提供）。

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や病原性にも違いが認められることから，別種として日本海裂頭条虫とされた[4]。両種はミトコンドリアDNAレベルでも異なる[5]。

日本海裂頭条虫の成虫は白色，扁平のキシメン様。体長 約10mにも及ぶ（図1a）。前端は細長い棍棒状の頭節があり，頸部，未熟片節，成熟片節，さらに子宮内に虫卵（図1b）が充満した受胎片節と続く。成熟片節の中央部には1対の雌雄生殖器を有す。ヒトに感染する幼虫（＝擬充尾虫，以下，プレロセルコイド）は体長約8mm，棍棒状で白色，伸縮性がある（図1c）。第2中間宿主であるサクラマスやシロザケなどサケ類の筋肉内に寄生する（図1d）[6]。日本海やオホーツク海沿岸からカムチャッカ，さらに北西太平洋沿岸地域に分布する。

一方，広節裂頭条虫の成虫，虫卵，プレロセルコイドの外形は日本海裂頭条虫のそれに似る。ユーラシア大陸と北米に分布するが，南米（チリ，アルゼンチン）には人為的な理由によって持ち込まれたと考えられている。日本海裂頭条虫と大きく異なるのは生活史にある。

2）生活史・ヒトへの感染経路

日本海裂頭条虫の生活史は未だ不明な点が多いが，サケ類が深く関与する（図2）。成虫はクマやヒトなど終宿主の小腸に寄生し，虫卵は糞便とともに排出される。虫卵は発育してコラシジウムになり，第1中間宿主（海産の動物性プランクトンと推定）に摂取され，前擬充尾虫（以下，プロセルコイド）になる。プロセルコイドに寄生された動物性プランクトンは食物連鎖によって第2中間宿主であるサクラマス，シロザケ，あるいはカラフトマスなど遡河回遊性のサケ類に摂取され，その筋肉内でプレロセルコイドに発育し，これがヒトへの感染型となる（図1c）。

広節裂頭条虫の生活史は淡水域で維持される。ヒト，イヌ，ネコの他，多くの野生哺乳動物が終宿主となり，糞便とともに外界に排出された虫卵は淡水で発育し，コラシジウムになる。その後，第1中間宿主であるケンミジンコの体内でプロセルコイドになり，さらに第2中間宿主の淡水産魚類でプレロセルコイドに発育する。欧州や北米ではカワカマス（＝ノーザンパイク）やスズキ目のパーチ[2]，南米ではニジマスやそれらの食餌となる小型の淡水魚（トウゴロウイワシ類）が第2中間宿主として知られている[7]。

3）疫学

裂頭条虫症は1970年代当初，欧州とアジアを中心に世界で900万人の感染者がいると推定されたが，最近，その数は疑問視されている[8]。わが国では，1889年の第1例以来，報告された症例数だけでも1,800例近くある[3]。かつては，サケ漁が盛んであった北日本で頻発していたが，1970年半ば以降，生鮮魚介類の輸送法の急速な進歩に伴い，北海道から沖縄まで全国で発生するようになり，年間の発生数は数百例と推定された[9]。近年，日本海裂頭条虫症は増加傾向にあるとされるが[10]，実際の発生数を把握することは困難である。

世界的には，かつて広節裂頭条虫症の発生が多かった北欧では，最近，減少傾向にある[11,12]。しかし，サケ・マス市場のグローバル化に伴うサケ・マス消費量の増加によって，今まで日本海裂頭条虫症が知られていなかった欧州[13-15]，北米[16]，あるいはニュージーランド[17]で感染した症例が報告されている。著者らは日本海裂頭条虫症が中国の大都市でも発生し始めたことを最

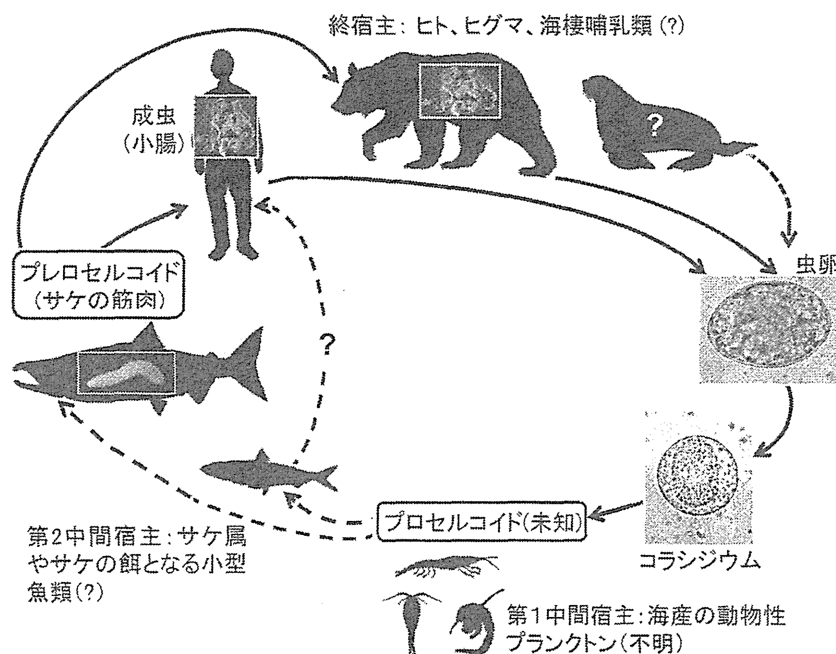

図2．日本海裂頭条虫の生活史。破線は推定される経路。