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Review
Introduction to the symposium on cestode zoonoses in Asia and the Pacific at the 21st Pacific Science Congress

Significance of Molecular Diagnosis using Histopathological Specimens in Cestode Zoonoses

Hiroshi Yamasaki[1,2,*], Kazuhiro Nakaya[3], Minoru Nakao[1], Yasuhito Sako[1] and Akira Ito[1]

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Abstract: Cestode zoonosis cases confirmed by PCR-based mitochondrial DNA analysis were investigated. The cestodiosis included taeniasis, cysticercosis, alveolar echinococcosis, cystic echinococcosis, sparganosis mansoni, diphyllobothriasis and diplogonoporiasis. DNA samples were extracted from the ethanol-fixed, formalin-fixed, paraffin-embedded sections, HE-stained, and the PAS- or acetocarmine-stained samples submitted for histopathology. For PCR-based analysis, cytochrome *c* oxidase subunit 1 and/or cytochrome *b* genes were amplified by multiplex PCR or conventional PCR coupled with DNA sequencing. Although DNA molecules were degraded in most formalin-fixed samples, smaller gene fragments were successfully amplified and the species causing cestodiosis could be identified by DNA sequence analysis of the amplicons. This review describes cestode zoonosis cases in which mitochondrial DNA analysis was useful not only for routine and retrospective diagnosis, but also for genetic polymorphism analysis and molecular identification of the species associated with pathogenicity. The significance of molecular diagnosis using histopathological specimens for cestode zoonoses is also discussed.
Key Words: Cestode zoonoses, mitochondrial DNA analysis, histopathological specimens

INTRODUCTION

The diagnosis of cestodiosis in humans, including cysticercosis, alveolar echinococcosis (AE), cystic echinococcosis (CE) and sparganosis mansoni is based upon clinical manifestations, imaging examinations, serology and histopathology. These diagnostic methods have advantages and limitations. Imaging findings using X-ray, computed tomography (CT), magnetic resonance imaging (MRI) and ultrasound (US) are useful for the diagnosis of cestode zoonoses, but are not always specific for each cestodiosis. Serological examinations are highly sensitive and useful in cysticercosis with multiple cysts, AE, CE, and sparganosis mansoni. However, the sensitivity decreases in cases of cysticercosis with a solitary cyst and obsolete calcified cysts [1-5]. Serology in cases of intestinal taeniasis, diphyllobothriasis and diplogonoporiasis is not usually performed. Histopathology provides confirmatory evidence for definitive diagnosis of the cestodiosis; however, it is occasionally difficult to identify the causative cestode species due to the degeneration and/or calcification of the lesions, particularly in cysticercosis cases (Table 1) [6-10]. Morphological identification is useful for taeniasis, diphyllobothriasis and diplogonoporiasis, but it is difficult to precisely identify the tapeworm species that cause the cestodiosis due to the morphological similarities.

To compensate for these diagnostic limitations, therefore polymerase chain reaction (PCR)-based molecular diagnosis has recently been introduced for the definitive diagnosis of cestode zoonoses [6, 7, 9-13]. More recently, DNA diagnosis using histopathological specimens has been applied to protozoan infections [14-16] and nematode infection [17]. In this review, cestodiosis cases in which the mitochondrial DNA analysis coupled with histopathology was absolutely useful for a definitive diagnosis are described, and the significance of molecular analysis in clinical diag-

[1] Department of Parasitology, Asahikawa Medical College, Asahikawa, Japan
[2] Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan
[3] Animal Laboratory for Medical Research, Asahikawa Medical College, Asahikawa, Japan

* Corresponding author
Tel: +81-3-5285-1111 ext. 2200
Fax: +81-3-5285-1173
E-mail: hyamasak@nih.go.jp

Table 1. Cestodiosis cases diagnosed by mitochondrial DNA analysis.

Cestodiosis	Case #	Year	Patient (age/sex/nationality/infection place)	DNA sources	Specimen condition	Target DNA	Diagnostic methods	Species identification	References
Taeniasis	1	2005	45/M/ Japanese/ Thailand	proglottid	ethanol-fixed	*cox1*	multiplex PCR/ DNA seq	*T. saginata*	[52]
	2	2005	28/F/ Thai/ Thailand	2 scolices with and without hooklets	acetocarmine-stained	*cox1/cob*	BESS T-base analysis	*T. solium* and *T. asiatica*	[28]
	3	2006	16/M/ Cambodian/ Cambodia	proglottid	ethanol-fixed	*cox1*	multiplex PCR/ DNA seq	*T. saginata*	This study
	4	2006	58/M/ Japanese/ South Korea ?	proglottid	ethanol-fixed	*cox1*	multiplex PCR/ DNA seq	*T. saginata*	This study
	5	2006	32/F/ Japanese/ Ethiopia	proglottid	ethanol-fixed	*cox1*	multiplex PCR/ DNA seq	*T. saginata*	This study
	6	2007	33/M/ Japanese/ Cambodia or Ethiopia	proglottid	ethanol-fixed	*cox1*	multiplex PCR/ DNA seq	*T. saginata*	This study
	7	2007	40/F/ Japanese/ not specified	proglottid	ethanol-fixed	*cox1*	multiplex PCR/ DNA seq	*T. saginata*	This study
Cysticercosis	1	2002	53/F/ Japanese/ Southeast Asia or India	brain tissue	formalin-fixed/paraffin sections*	*cox1*	PCR/ DNA seq	*T. solium* (Asian type)	[12]
	2	2003	9/F/ Filipino/ Philippines	brain tissue	formalin-fixed/paraffin sections*	*cox1/cob*	PCR/ DNA seq	*T. solium* (Asian type)	[6]
	3	2003	83/M/ Japanese/ China	calcified cysts from muscle	formalin-fixed/paraffin sections*	*cox1*	nested PCR/ DNA seq	*T. solium* (Asian type)	[7,32]
	4	2005	87/M/ Japanese/ Okinawa, Japan	calcified cysts from muscle	formalin-fixed/paraffin sections*	*cox1/cob*	nested PCR/ DNA seq	*T. solium* (Asian type)	[10]
	5	2005	28/F/ Indian/ India	brain tissue	formalin-fixed/paraffin sections*	*cox1/cob*	PCR/ DNA seq	*T. solium* (Asian type)	[33]
	6	2005	24/F/ Japanese/ Bali, Indonesia	brain tissue	formalin-fixed/paraffin sections*	*cox1*	PCR/ DNA seq	*T. solium* (Asian type)	[13]
	7	2005	38/F/ Japanese/ Nepal	brain tissue	formalin-fixed/paraffin sections*	*cox1*	PCR/ DNA seq	*T. solium* (Asian type)	[9]
	8	2006	42/F/ Brazilian/ Brazil	brain tissue	formalin-fixed/paraffin sections*	*cox1/cob*	nested PCR/ DNA seq	*T. solium* (American/African type)	[34]
Alveolar echinococcosis	1	1978	62/F/ Japanese/ Fukui, Japan	liver tissue	formalin-fixed/paraffin sections*	*cox1*	PCR/ DNA seq	*E. multilocularis*	[35]
	2	1982	73/F/ Japanese/ Hokkaido, Japan	liver tissue	HE-stained sections	*cox1*	nested PCR/ DNA seq	*E. multilocularis*	[8]
	3	1992	62/F/ Japanese/ Hokkaido, Japan	liver tissue	formalin-fixed/paraffin sections*	*cox1*	nested PCR/ DNA seq	*E. multilocularis*	[8]
	4	1993	?/F/ Japanese/ Hokkaido, Japan	liver tissue	formalin-fixed/paraffin sections*	*cox1*	nested PCR/ DNA seq	*E. multilocularis*	[8]
	5	2004	48/F/ Japanese/ Hokkaido, Japan	liver tissue	formalin-fixed/paraffin sections*	*cox1*	PCR/ DNA seq	*E. multilocularis*	[112]
	6	2006	64/F/ Japanese/ Hokkaido, Japan	liver tissue	ethanol-fixed	*cox1*	PCR/ DNA seq	*E. multilocularis*	[113]
	7	2006	47/F/ Japanese/ Hokkaido, Japan	liver tissue	PAS-stained sections	*cox1*	PCR/ DNA seq	*E. multilocularis*	[113]
Cystic echinococcosis	1	2004	27/M/ Peruvian/ Peru	cyst fluid containing protoscolices	ethanol-fixed	*cox1*	PCR/ DNA seq	*E. granulosus* (G1)	[11]
	2	2004	20/F/ Peruvian/ Peru	cyst fluid containing protoscolices	ethanol-fixed	*cox1*	PCR/ DNA seq	*E. granulosus* (G1)	[38]
	3	2004	40/F/ Peruvian/ Peru	cyst fluid containing protoscolices	ethanol-fixed	*cox1*	PCR/ DNA seq	*E. canadensis* (G6)	[39]
	4	2005	36/M/ Iranian/ Iran	cyst fluid containing protoscolices	ethanol-fixed	*cox1*	PCR/ DNA seq	*E. granulosus* (G1)	[40]
	5	2007	30/M/ Uzbek/ Uzbekistan)	cyst	ethanol-fixed	*cox1*	PCR/ DNA seq	*E. granulosus* (G1)	[41]
	6	2007	29/M/ Japanese/ Peru ?	cyst fluid containing protoscolices	frozen	*cox1*	PCR/ DNA seq	*E. granulosus* (G1)	[42]
Sparganosis	1	2002	64/M/ Japanese/ Nara, Japan	plerocercoid	ethanol-fixed	*cox1*	PCR/ DNA seq	*S. erinaceieuropaei*	[47]
	2	2005	67/F/ Japanese/ Nagano, Japan	plerocercoid	frozen	*cox1*	PCR/ DNA seq	*S. erinaceieuropaei*	[8]
	3	2006	58/F/ Japanese/ Aichi, Japan	plerocercoid	formalin-fixed	*cox1*	PCR/ DNA seq	*S. erinaceieuropaei*	[8]
Diphyllobothriasis	1	2006	4/M/ Japanese/ Aichi, Japan	strobila	formalin-fixed/paraffin sections*	*cox1*	Nested PCR/ DNA seq	*D. nihonkaiense*	[8]
	2	2006	3/F/ Japanese/ Aichi, Japan	2 strobilae	formalin-fixed/paraffin sections*	*cox1*	Nested PCR/ DNA seq	*D. nihonkaiense*	[8]
	3	2006	52/M/ Japanese/ New Zealand	strobila	ethanol-fixed	*cox1*	PCR/ DNA seq	*D. nihonkaiense*	in preparation
	4	2007	44/F/ Japanese/ Saitama, Japan	strobila	ethanol-fixed	*cox1*	PCR/ DNA seq	*D. nihonkaiense*	This study
	5	2007	44/F/ Japanese/ Tokyo, Japan	strobila	ethanol-fixed	*cox1*	PCR/ DNA seq	*D. nihonkaiense*	This study
Diplogonoporiasis	1	2006	58/M/ Japanese/ Hamamatsu, Japan	strobila	ethanol-fixed	*cox1*	PCR/ DNA seq	*D. grandis (=D. balaenopterae ?)*	[52]
	2	2006	58/M/ Japanese/ Tokyo, Japan	strobila	ethanol-fixed	*cox1*	PCR/ DNA seq	*D. grandis (=D. balaenopterae ?)*	This study

* unstained sections

nosis and parasitological studies is discussed.

MATERIALS AND METHODS

Cestode samples examined

The cestodiosis cases presented here were received with requests for diagnosis from domestic and overseas medical institutions between 2001 and 2007 (Table 1). The total number of samples examined was 38 from 36 patients. Of these, there were 5 diagnoses of taeniasis, 8 of cysticercosis, 7 of AE, 6 of CE, 3 of sparganosis mansoni, 5 of diphyllobothriasis, and 2 of diplogonoporiasis. Most samples were obtained over the last 7 years, but 4 samples of AE (cases 1-4, Table 1) were archival specimens prepared 15～29 years earlier. Parasite samples of taeniasis, CE, and some diphyllobothriasis and diplogonoporiasis cases were properly preserved in 80% ethanol for DNA analysis, but samples from cysticercosis and AE cases were 10% formalin-fixed and processed to paraffin-embedded unstained sections (10-μm in thickness). As trials, acetocarmine (case 2 of taeniasis), hematoxylin-eosin (HE)(case 2 of AE), and periodic acid/Schiff (PAS)-stained sections (case 7 of AE) attached to the slide glasses were also examined for molecular analysis.

Mitochondrial DNA analysis

DNA samples from ethanol-fixed cestode samples and cyst fluids from CE patients were prepared using a DNeasy tissue kit (Qiagen, Germany). DNA samples from 10% formalin-fixed paraffin-embedded sections were prepared using commercially available kits such as DEXPAT (TaKaRa Bio., Japan) or a DNA Isolator PS kit (Wako Pure Chemicals, Japan). DNA samples from the HE- and PAS-stained sections mounted onto slide-glasses were prepared using a DNA Isolator PS kit. In cases of very limited sample quantity (for instance, case 3 of sparganosis), 0.05 N NaOH solution was used for DNA extraction at 95-100℃. The amplification of target genes, the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) and/or cytochrome *b* gene (*cob*), was performed according to the protocols previously described [6, 11, 18, 19].

For materials fixed in 10% formalin, primer pairs that can amplify 100～200 base pairs (bp) fragments were utilized [6, 7, 9, 10, 13] because of DNA degradation due to cross-linking with formalin [20]. Nested PCR was performed depending on the quantity of the samples, particularly the paraffin-embedded sections, because of the probably low extraction efficiency of DNA from such samples. In order to confirm whether DNA was extracted from the paraffin-embedded tissue section, a microsatellite marker of the TH01 locus in the human tyrosine hydroxylase gene (180-190 bp) was amplified as a control [6, 7, 21].

The PCR-amplified products were subjected to electrophoresis in 1% agarose gels, 4-20% gradient polyacrylamide gels or a capillary electrophoresis system. The samples for DNA sequencing were prepared using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kits (ABI PRISM, version 1.1 or 3.3, USA). DNA sequencing was performed on an ABI PRISM 310 or 3100 Genetic Analyzer (Applied Biosystems, USA).

RESULTS

Since most of the tissue or parasite samples had been fixed in 10% formalin prior to molecular analysis, only smaller target gene fragments were amplified. The two kits used to extract DNA from paraffin-embedded sections yielded similar results in the amplification of target genes. For DNA analysis, therefore, it is strongly recommended that parasite samples be preserved in >80% ethanol to prevent the DNA degradation that occurs during formalin fixation.

Taeniasis

Taenia saginata and *Taenia solium* are well documented as cestodes causing taeniasis in humans. In addition, *Taenia asiatica*, which is morphologically close to *T. saginata*, can also cause taeniasis in humans; it is distributed in Asia [22-24] including China [25, 26], Vietnam [27], the Philippines [25], Thailand [28], Malaysia [25], and Indonesia [5, 22-25, 29, 30]. These 3 taeniid cestodes are sympatrically distributed in certain Asian regions [28], implying that accurate differentiation of these species is important for case reports, as well as prevalence and epidemiological studies in the affected areas. With the recent increase in people travelling abroad and immigrants from endemic areas, taeniasis as an imported parasitic disease has been reported sporadically in Japan [31]. Indeed, of the 5 taeniasis cases presented here, all except for case 2 were imported. Since it is difficult to differentiate these taeniid cestodes because of their morphological similarity, molecular diagnosis is useful. Thus, in order to identify the taeniid species accurately, differential diagnosis using BESS T-base analysis and multiplex PCR are very useful and informative [18, 19].

Fig. 1 shows the molecular identification of 4 proglottids from case 4 by multiplex PCR. This is a method that can differentiate the taeniid cestodes accurately by the size of the PCR products [19]. The taeniid species were identified as *T. saginata* based on the 827-bp *cox1* gene fragments (Fig. 1B), and the result was supported by DNA sequencing (Fig. 1C). Taeniasis case 2 provides an example

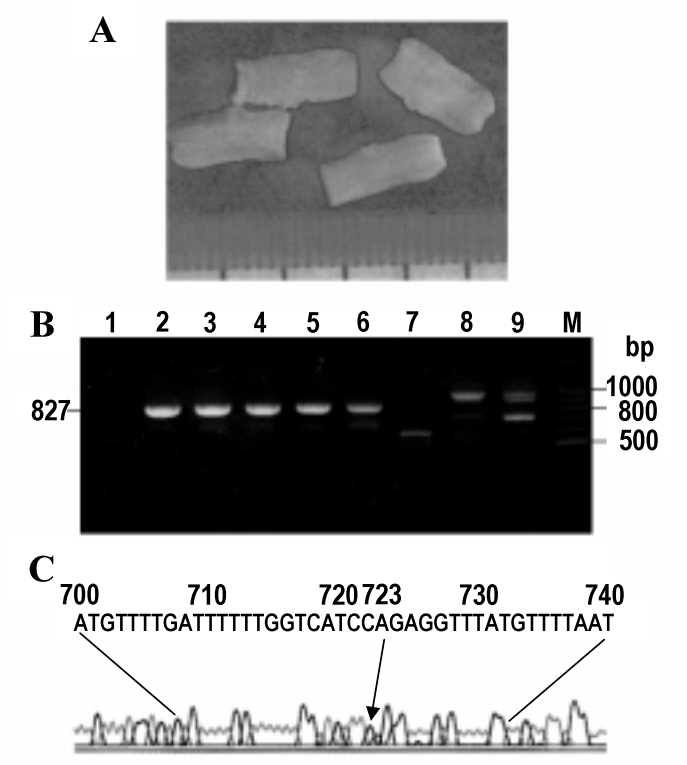

Fig. 1. Molecular identification of taeniid proglottids by multiplex PCR.
A: 4 proglottids expelled naturally in case 4. B: *cox1* products amplified by multiplex PCR from individual proglottid. The 827 -bp products are diagnostic in size for *T. saginata* (lanes 2-5). Lane 1, blank without DNA template; lanes 6-9, *T. saginata* (827 bp) *T. asiatica* (588 bp), *T. solium* (Asian genotype, 984 bp), and *T. solium* (American/African genotype, 720 bp) as controls, respectively. The products were run in a 1% agarose gel. M, 100-bp ladder DNA size markers (Promega). C: DNA sequencing of the amplicons. The nucleotide at position 723 of the *cox1* gene is adenine, which is a unique nucleotide marker for *T. saginata*.

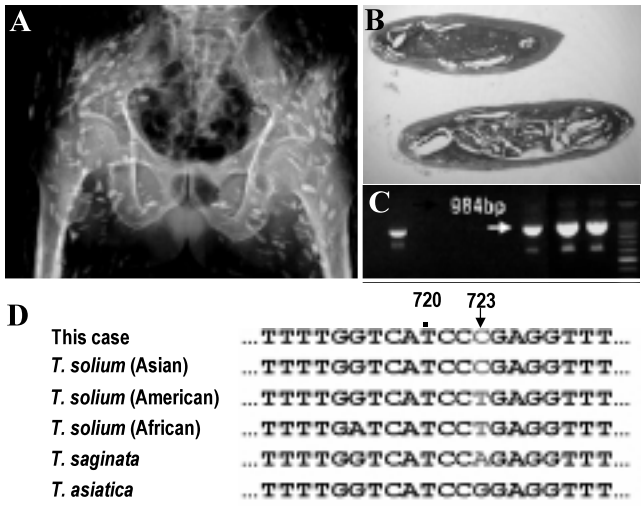

Fig. 2. Molecular diagnosis of cysticercosis with calcified cysts in case 3.
A: X-ray findings showing numerous calcified cysts scattered in the systemic muscles. B: histopathological findings of biopsied cyst specimens. C: PCR-amplified 984-bp *cox1* gene fragments from 3 resected calcified cysts. The products were run in a 1% agarose gel. D: comparison of the nucleotide sequences of PCR-amplified *cox1* with other sequences from *T. solium* (Asian genotype, AB 066486), *T. solium* (American/African genotype, AB 066492), *T. saginata* (AB 066495) and *T. asiatica* (AB 066494). The nucleotide at position 723 is cytosine, indicating the causative *T. solium* is the Asian genotype.

of the difficulty of morphological identification. Two scolices with and without hooklets expelled from a Thai taeniasis patient were identified morphologically as *T. solium* and *T. saginata*, respectively. However, the taeniid cestode without hooklets was identified as *T. asiatica* by mitochondrial DNA analysis [28], implying that molecular identification is indispensable for precise identification of taeniid cestodes that resemble each other morphologically. Also, acetocarmine-stained materials were available for DNA analysis, although the DNA was fragmented by HCl treatment.

Cysticercosis

We encountered 8 *T. solium* cysticercosis cases confirmed by mitochondrial DNA analysis using the biopsy specimens. As shown in Table 1, 5 cysticercosis cases (2, 3, 4, 6, 7) presented no histopathological findings and produced negative results upon serology, although case 5 with multiple cysts was sero-positive. Subsequently, the 5 cases were diagnosed as cysticercosis by molecular analysis using the histopathological specimens. In case 1, 1.6-kb *cox1* and 1.3-kb *cob* gene fragments were successfully amplified even though the specimen had been fixed in formalin [12]. However, smaller target gene fragments were amplified in the other 7 cases. In case 2, 93～374-bp *cox1* and 126～191-bp *cob* gene fragments were amplified [6].

In two cases of intramuscular cysticercosis with numerous calcified cysts in the muscles (Fig. 2A), the histopathological findings of the extremely calcified cysts were not defined (Fig. 2B). However, a 984-bp gene fragment was amplified from the samples fixed in formalin (Fig. 2C), identifying the cysts as being due to the *T. solium* Asian genotype (Fig. 2D)[7], whereas smaller 224-bp *cox1* fragments were amplified in 3 cystic lesions from case 4. Again, it was found that the calcified cysts were derived from an Asian genotype of *T. solium* [10]. The patient in case 3 had a history of residence in northeast China approximately 60 years ago [32] while the patient in case 4 lives in Okinawa [10], which was once endemic for cysticercosis. In an Indian patient (case 5), neurocysticercosis with multiple cysts

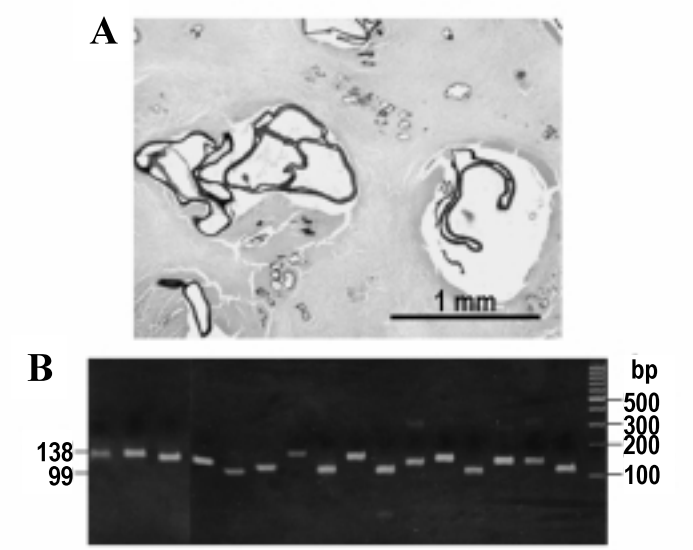

Fig. 3. Molecular analysis of the *cox1* gene from *E. multilocularis* using an archival specimen.
A: histopathological findings showing characteristic laminated layers in case 1 (PAS-stain). B: 16 overlapping *cox1* gene fragments (99～131 bp) amplified by PCR from the archival specimen in case 1. The products were run in a 4-20% gradient polyacrylamide gel.

was highly suspected based on the serology and imaging findings, although the histopathological findings were not supportive due to the degeneration of the cystic tissues. DNA analysis identified the causative agent as the *T. solium* Asian genotype [33].

The patient in case 6 had repeated epileptic seizures and was diagnosed definitively with cysticercosis by histopathology [13]. The patient had a history of repeated travels to 11 countries between 1999 and 2005, including Korea, Indonesia (Bali) and Mexico where cysticercosis remains endemic. It was originally thought that the patient was infected with *T. solium* in Mexico, based on her latest travel history. However, *cox1* gene analysis using paraffin-embedded sections revealed that the patient was exposed to *T. solium* in Bali, Indonesia based on a unique nucleotide conserved among Bali isolates of *T. solium* [30]. Case 7 could not be confirmed histopathologically as cysticercosis, but DNA of the *T. solium* Asian genotype was detected [9]. In case 8, histopathology suggested a racemose-type cysticercosis with hydrocephalus [34], and molecular analysis using the histopathological paraffin-embedded sections confirmed the cysticercus to be an American/African genotype of *T. solium*. This patient was from Brazil.

Alveolar echinococcosis

The diagnosis of AE caused by *Echinococcus multilocularis* metacestodes is obtained by US, CT and MRI. Serology and histopathology are also useful and reliable diagnostic methods, so molecular diagnosis may not be essential for a definitive diagnosis. However, molecular pathology for AE is useful for the differential diagnosis of other diseases such as infectious liver cyst that present similar pathological findings. The analysis of genetic polymorphism among *E. multilocularis* isolates obtained worldwide is also an interesting subject that requires molecular analysis.

Of 7 AE cases reported here (Table 1), 6 were from Hokkaido, the northern island of Japan where AE is still endemic. The other (case 1) was from Fukui City, situated in the mid-western region of Honshu, the main island of Japan, a area remote from Hokkaido; this case was diagnosed as an autochthonous AE at autopsy in 1979 [35]. For case 1, an archival unstained paraffin-embedded section (2 x 3 cm) prepared approximately 30 years ago was used to genotype *E. multilocularis* (Fig. 3A). DNA was extractable from the section, but 16 smaller fragments (99～131 bp) covering the *cox1* gene (1,608 bp) were successfully amplified (Fig. 3B). By combining these 16 overlapping gene fragments, the complete nucleotide sequence of the *cox1* gene proved that the *E. multilocularis* was identical to *E. multilocularis* isolates from Hokkaido, although it remains unclear how the patient had been infected with *E. multilocularis* in such a remote area. For cases 3-6, unstained paraffin-embedded sections were used, and small *cox1* gene fragments (<220 bp) were amplified. In case 2, for which a HE-stained specimen was used, only a 220-bp *cox1* gene fragment was amplified, whereas 220- and 410-bp *cox1* gene fragments were detected in the PAS-stained specimens available for case 6. However, the amplification of a longer *cox1* fragment (825 bp) was not successful. In case 7, for which 80% ethanol-fixed liver tissue was used, a >1.6 kb *cox1* gene was easily amplified. These results demonstrate that HE- and PAS-stained sections are also potential materials for use in retrospective studies because many histopathological specimens are stained with HE and PAS.

Cystic echinococcosis

Cystic echinococcosis is caused by the infection of larval *Echinococcus granulosus*, but the taxonomy of the *Echinococcus* species has been a controversial issue for decades. Thompson and McManus [36] proposed that the number of recognized species of *E. granulosus* (genotypes, G1～G10) is 4 to 6. Most recently, a taxonomic revision of *Echinococcus* species based on complete mitochondrial genome analysis was conducted [37]. According to the revision, *E. granulosus* is classified into at least 4 distinct species: *E. granulosus* (G1, G2 and G3), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5), and *Echinococcus canadensis* (G6, G7, G8), although the buffalo, lion and

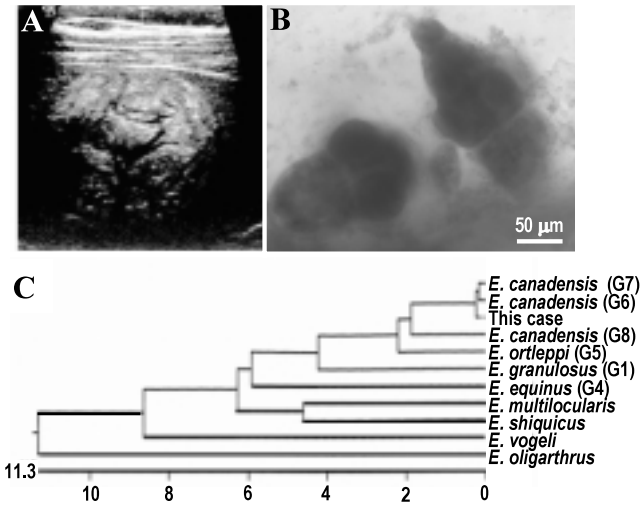

Fig. 4. Molecular identification of the *Echinococcus* species causing cystic echinococcosis.

A: ultrasonographic findings showing a peculiar soccer ball-appearance with hypoechoic lamination and a number of small hyperechoic foci (modified from [39]). B: protoscolices found in aspirated cyst fluid. C: phylogenetic tree based on the *cox1* nucleotide sequence.

Fennoscandian cervid (G10) strains have not been analyzed.

We have carried out genotyping of *Echinococcus* species associated with pathogenicity when cystic lesions from CE patients were available. Over the last 4 years we encountered 6 CE cases consisting of 3 Peruvian [11, 38, 39] and 1 Iranian [40], Uzbek [41] and Japanese [42] patients (Table 1). The parasite samples from all cases except for case 5 were cyst fluids including protoscolices aspirated by the puncture-aspiration-injection-reaspiration (PAIR) method. In case 1, imaging findings presented 2 cystic lesions in the liver and a giant cyst in the abdominal cavity [11]. The patient in case 2 was found by US to have 2 cystic peculiar soccer ball-like lesions in the liver (Fig. 4A). Many protoscolices were found in the cyst fluid aspirated by PAIR (Fig. 4B). Since parasite samples from all CE patients were preserved properly for DNA analysis, diagnostic 798-bp *cox1* gene fragments (positions 324-1,121) were stably amplified. As shown in Table 1, the *Echinococcus* species causing CE, except for case 3, were all identified as *E. granulosus* (sheep strain, G1) by mitochondrial DNA analysis. In case 3, however, the causative species was identified as *E. canadensis* with high sequence homology (99.6～99.8% versus G6) and clearly distinguished from *E. granulosus* (Fig. 4C). The *E. granulosus* complex is distributed worldwide, and it is expected that CE patients will be found sporadically in association with the recent increase in immigrants from Peru, the Middle East and other areas where CE is highly endemic.

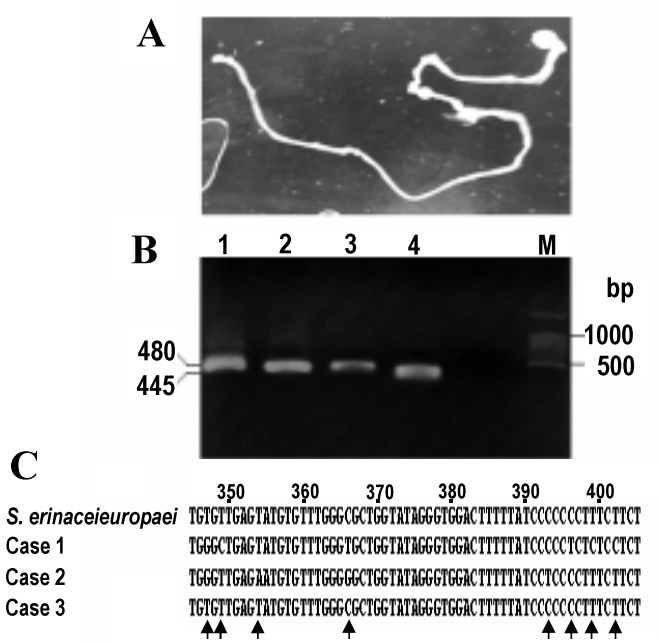

Fig. 5. Analysis of genetic polymorphism of *Spirometra* plerocercoids.

A: a plerocercoid resected from case 3. B: PCR-amplified 4 *cox 1* gene fragments covering the complete *cox1* gene. M, 100-bp ladder DNA size markers (Promega). C: genetic polymorphism of *cox1* among *Spirometra* plerocercoids. *S. erinaceieuropaei cox1* is from AB 015754. Arrows indicate mutated nucleotides.

Sparganosis mansoni

Sparganosis mansoni is a cestodiosis caused by an infection of larval *Spirometra* plerocercoids, and while distributed worldwide, it occurs mainly in Asia. There were approximately 400 human cases reported up to 1996 in Japan [43], and new cases have been reported annually [44-46]. Histopathology for sparganosis is highly confirmatory if the plerocercoid is surgically removed from a patient presenting a creeping eruption or formation of a subcutaneous tumor-like lesion. Serology is also useful since strong immune responses are induced by infection. In Japan, *Spirometra erinaceieuropaei* has been identified as the species causing sparganosis mansoni whereas *Spirometra mansonoides* is the main species in North and South America. Furthermore, little is known about intraspecific genetic variations in *S. erinaceieuropaei* and *S. mansonoides* isolates.

Therefore, in order to identify the plerocercoid species accurately and to examine intraspecific genetic polymorphism, the accumulation of molecular data is needed. The plerocercoid samples from cases 1 [47] and 3 [8] were fixed in 10% formalin (Fig. 5A), but 4 small fragments (445～480 bp) covering the complete *cox1* gene (1,566 bp) were successfully amplified (Fig. 5B). A 1.6-kb *cox1* gene was amplified from a frozen plerocercoid from case 2 (data not

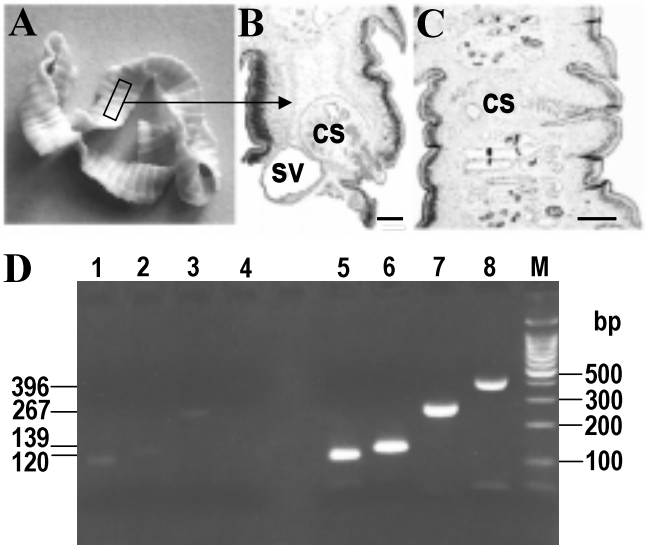

Fig. 6. Molecular identification of diphyllobothriid tapeworms expelled from patients infected after eating raw salmons.
A: a strobila expelled from case 1. B: sagittal sections of boxed area in A. In this section, the cirrus sac (cs) is situated obliquely against the seminal vesicle (sv), indicating the feature of *D. nihonkaiense*. C: sagittal section of another strobila from case 2. The position of the cirrus sac is suggestive of *D. latum*. Bars = 100 μm. D: PCR-amplified 120～396-bp *cox1* gene fragments in a 1% agarose gel (case 1). Lanes 1-4, 1st PCR products. Lanes 5-8, nested PCR products. M, 100-bp ladder DNA size markers (Promega).

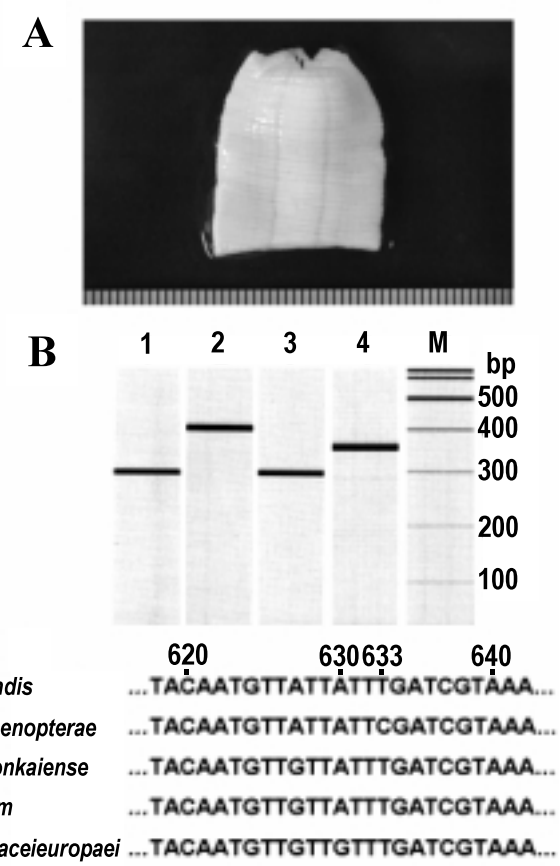

Fig. 7. Phylogenetic relationship between *D. grandis* and *D. balaenopterae*.
A: a part of the strobila used as a DNA source from case 1. B: PCR-amplified *cox1* products run using a capillary electrophoresis system (HAD-GT 12, Gene Inc.). Lanes 1-4, positions 1-300, 501-900, 901-1,200 and 1,201-1,566 of the *cox1* gene, respectively. C: comparison of the nucleotide sequences of *cox1* genes from *D. grandis*, *D. balaenopterae* (Kuramochi, personal communication), *D. nihonkaiense* (AB 268585), *D. latum* (AB 269325) and *S. erinaceieuropaei* (AB 015754).

shown). Subsequent DNA sequence analysis identified all of the plerocercoids as *S. erinaceieuropaei* with slight intraspecific variations of 1.1～2.4% (Fig. 5C).

Diphyllobothriasis

Diphyllobothriasis is a fish-borne cestodiosis caused by the infection of adult pseudophyllidean tapeworms *Diphyllobothrium* spp.[48]. Ingesting the plerocercoid in raw or undercooked fish infects humans. Out of 37 diphyllobothriid species, 11 species have been reported as agents causing diphyllobothriasis in humans [43]. In Japan, *D. nihonkaiense*, *D. yonagoense*, *D. cameroni*, *D. scoticum*, *D. hians*, *D. orcini*, *D. pacificum* and *D. latum* have been reported as species causing human diphyllobothriasis [43]. The name *D. latum* was used for the main species causing human diphyllobothriasis for over a century in Japan; however, *D. nihonkaiense* was newly described as a species causing diphyllobothriasis in Japan [49], and has recently been recognized as diphyllobothriasis nihonkaiense. A prominent differential criterion for *D. nihonkaiense* and *D. latum* is the position of the cirrus sac and seminal vesicle: the cirrus sac is situated obliquely against the anterior-posterior axis in *D. nihonkaiense*, whereas it is situated horizontally in *D. latum* [43]. However, this morphological criterion is apparently not absolute due to contraction of the tapeworms upon fixation or the position of the segments to be sectioned.

We have encountered 5 cases of diphyllobothriasis over the past two years (Table 1). Figs. 6A-C show a diphyllobothriid strobila expelled from a Japanese infant (case 1) and sagittal sections of the 2 diphyllobothriid tapeworms expelled from cases 1 and 2. The cestode (Fig. 6B) appears to be *D. nihonkaiense* because the cirrus sac is situated obliquely, but the other (Fig. 6C) shows morphology resembling *D. latum*. Upon molecular analysis using paraffin-embedded sections prepared from these strobilae, 120～396-bp *cox1* gene fragments were amplified (Fig. 6 D), and DNA sequence analysis of the 396-bp *cox1* gene fragments revealed all the diphyllobothriid tapeworms to be

Table 2. *T. solium* cysticercosis cases in HIV-infected patients.

Patient (age /sex /nationality)	Clinical findings	References
22/F / Haitian	cocurrent infection with toxoplasmosis, encephalitis and tuberculous abscess	[55]
40/M / Zimbabwean	hydrocephalus, contiguous lesions in the right frontal lobe, **racemose-type**	[56]
30/M / Zimbabwean	numerous cysts in right posterior limb	[56]
36/M / Zimbabwean	multiple cysts in the right frontal cortex	[56]
25/M / Zimbabwean	multiple cysts in the cortex	[56]
29/M / Mexican	multiple cysts in the occipital lobe without surrounding edema	[57]
29/M / Mexican	**a giant mass** in the right frontoparieto-temporal region	[58]
41/F / Mexican	a round lesion in the right basal ganglia, **racemose-type**	[58]
32/M / Indian	a subretinal, translucent white cyst in the fundus of the left eye with a central dense white spot; cocurrent infection with AIDS pulmonary tuberculosis, oropharyngeal candidiasis, *Pneumocystis carinii* pneumonia, and cryptococcal meningitis	[59]
45/M / Haitian	two cysts in the right and left frontal lobes, **racemose-type**	[60]
22/F / Ecuadorian	multiple cysts in parenchymal, ventricular and subarachnoidal regions	[61]
51/F / Indian	a solitary 14-mm rim-enhanced lesion in the right parietal lobe with a hyperintense mural nodule	[62]
40/M /Honduran	numerous cysts in the right and temporofrontal lobes	62]
72/M / Peruvian	numerous lesions in the intraparenchymal region, some with a mural nodule	[62]
56/F / Ethiopian	numerous ring-enhanced lesions in the brain; cocurrent infection with neurotoxoplasmosis and pulmonary tuberculosis	[63]

D. nihonkaiense with 99.7% sequence homology. The first 2 patients had eaten raw Masu salmon, *Oncorhynchus masou*, caught in Toyama Prefecture, in June 2006. Case 3 was an interesting case in which the patient was believed to have been infected with *D. nihonkaiense* after eating raw sliced salmon in New Zealand (Yamasaki et al., in preparation). In case 4, the infection source was not specified, but the patient in case 5 had eaten "*masu-zushi*" made from raw salmon from Toyama Prefecture, probably *O. masou*. In any event, the diphyllobothriids from cases 1-5 were all identified as *D. nihonkaiense* at the DNA level.

Diplogonoporiasis is also a fish-borne cestodiosis caused by an infection of the adult broad tapeworm, *Diplogonoporus grandis*, although the life cycle has not yet been completely clarified. More than 270 human cases were reported in Japan up to 1996 [50]. In Shizuoka Prefecture, a mass infection involving 46 patients occurred during the period from May to September 1996 [51]. With regard to the patient in case 1, a 6-m strobila expelled after treatment was identified as *D. grandis* due to its one-paired gonads. Fig. 7 A shows part of the strobila used for DNA analysis. Similarly, in case 2, a strobila was expelled and identified morphologically as *D. grandis*. The strobilae from these cases were preserved in 80% ethanol, and 4 *cox1* gene fragments covering the complete *cox1* gene (1,566 bp) were amplified by PCR (Fig. 7B). The nucleotide sequences of the *cox1* genes from the two *D. grandis* from cases 1 and 2 were identical, and showed 99.9% sequence homology with *Diplogonoporus balaenopterae*, a tapeworm of Minke Whale (*Balaenoptera acutorostrata*) and Sei Whale (*Balaenoptera borealis*)(Fig. 7C). Whether *D. grandis* and *D. balaenopterae* are the same species or not remains a taxonomic issue to be resolved at the DNA level [43]. The data shown here suggest that *D. grandis* is a synonym for *D. balaenopetrae* [52]. At present, further analyses using mitochondrial and nuclear DNA are underway.

DISCUSSION

In this review, the authors stress the importance of molecular diagnosis for cestode zoonoses in cases confirmed by mitochondrial DNA analysis using histopathological specimens over the past 7 years. With the increase in people travelling abroad or coming from abroad, and advances in international transport systems for foods, reports of clinical cases as emerging and re-emerging infectious diseases or imported parasitic diseases have been increasing.

In Japan, there were at least 51 taeniasis cases reported up until 2003, but most cases were imported cases due to *T. saginata* infection; so far, no *T. asiatica* infections have been found [31]. However, it should be noted that taeniasis due to *T. asiatica* is distributed in Asia. A differential diagnosis of the taeniasis using DNA from proglottids is highly useful [5, 18, 19, 23, 24, 31, 53, 54]. Epidemiological investigations in endemic areas where these taeniid species are distributed sympatrically are also needed [22-24, 28].

The serological diagnosis for *T. solium* cysticercosis is occasionally not sensitive. More than half of cases of solitary neurocysticercosis and inactive cysticercosis with calcified lesions are expected to be sero-negative [1-4]. In such cases, therefore, it is important to obtain crucial DNA confirmation for a definitive diagnosis [5, 6, 19, 53, 54]. In ad-

Table 3. Cysticercosis cases in humans caused by zoonotic taeniid species other than *T. solium*.

Taeniid species	Patient (age /sex /nationality)	References
T. crassiceps	17/F / Canadian	[64]
T. taeniaeformis	77/M / Czekoslovak	[65]
T. crassiceps	33/M / German/ **AIDS**	[66]
T. crassiceps	15/F / German	[67]
T. crassiceps	33/M / French/ **AIDS**	[68]
T. crassiceps	38/F / American	[69]
T. crassiceps	38/M / French/ **AIDS**	[70]
T. crassiceps	34/M / French/ **AIDS**	[71]
T. taeniaeformis	7/F / Sri Lankan	[72]

dition, there have been concurrent infections with *T. solium* and human immunodeficiency virus (HIV) reported (Table 2)[53-62] because of the increasing frequency of HIV infection in areas in which cysticercosis is endemic [63]. Little is known about the influence of HIV infection on the frequency and clinical course of cysticercosis. In HIV-infected patients, neurocysticercosis with giant cysts and racemose cysticerci seems to be much more frequent, probably due to uncontrolled parasitic growth due to an impaired cell-mediated immune response [60]. Furthermore, cysticercosis cases caused by zoonotic *Taenia crassiceps* have been reported in patients with acquired immunodeficiency syndrome (AIDS) (Table 3)[53, 54, 64-72]. Thus, in order to diagnose these unusual cases definitively, molecular diagnosis is indispensable in the future if biopsy specimens are available [5, 8, 53, 54].

Interestingly, more recent work has shown that the number of hooks in *T. solium* developing in non-obese diabetic/Shi-severe combined immunodeficiency (NOD/Shi-*scid*) mice varies widely from none to 28 [73]. The variable formation of hooklets in 135-day-old *T. solium* metacestodes has also been reported [74], suggesting that the presence of hooklets is not always a key marker for the identification of *T. solium*. These facts indicate the increasing need for molecular diagnosis.

In Japan, 6-25 cases of human AE cases were reported annually between 1999 and 2007 [75]. The diagnosis depends on serology, imaging findings and the presence of tortuous laminated layers characterized by histopathology. However, other hepatic diseases, including liver cyst [76] and infectious liver cyst, that present similar histopathological and imaging findings need to be differentiated from AE. Molecular analysis using histopathological specimens can provide a differential diagnosis. As mentioned with regard to cases 2 and 7 of AE, HE- and PAS-stained specimens as DNA sources were available, although the DNA was degraded, indicating that molecular analysis using stained specimens is useful for the analysis of genetic polymorphism.

One or 2 human cases of CE were reported annually between 1999 and 2007 [75], including 5 of the cases listed here [38-42]. Of the 6 cases, 5 were imported infections from Peru, the Middle East and Central Asia where CE remains endemic. The sole Japanese patient (case 6) had traveled abroad (Peru, Bolivia, Australia, China etc), and is thought to have been exposed to *E. granulosus* during a stay in Peru. *Echinococcus* species that cause CE are distributed worldwide, but the species that infect humans are *E. granulosus* (G1, G2), *E. ortleppi* (G5) and *E. canadensis* (G6, G7, G8) according to the latest taxonomy [37], while the infectivity of *E. equinus* (G4), buffalo strain (G3), lion strain (G9), and Fennoscandian cervid strain (G10) to humans has not yet been confirmed. With the increasing number of immigrants from endemic areas, it is expected that CE patients will be found more frequently. In this sense, molecular analysis is indispensable not only for studying *Echinococcus* species associated with pathogenicity and clinical manifestations, but also for molecular epidemiology [11]. Medical doctors should be aware of CE when diagnosing conditions in people from overseas.

The *Spirometra* species causing sparganosis mansoni have been identified as *S. erinaceieuropaei* and *S. mansonoides* in Asia, and North and South America, respectively. It is difficult to differentiate *S. erinaceieuropaei* from *S. mansonoides* morphologically [43], although it has been reported that isozyme profiles are useful for differentiating these species [77]. Most recently, it has been reported that intraspecific variation of *S. erinaceieuropaei* based on *cox1* gene analysis is less than 2.6% [78], and our results are consistent with this report. It may be necessary to revise the taxonomic relation of these two species based on DNA analysis as well as intraspecific genetic variations.

Diphyllobothriasis due to *D. latum* is one of the most common forms of fish-borne cestodiosis in Europe and North America [79], whereas *D. nihonkaiense* is the main cause of human diphyllobothriasis in Japan. As many as 1,736 diphyllobothriasis cases including *D. latum* infections were reported in Japan up to 2000 [80], and new cases are still being reported. Historically, the name *D. latum* was used for human diphyllobothriid infections for over a century in Japan, making it very difficult to determine with any degree of certainty whether reported *D. latum* infections were in fact due to that tapeworm. It has been demonstrated that *D. nihonkaiense* and *D. latum* are distinct species based on morphology [49], isozyme profiles [81], immunoelectrophoresis patterns [82], ribosomal DNA sequences [83], and, most recently, mitochondrial genome analysis [84]. Nevertheless, *D. latum* infections acquired through

eating raw salmons are still reported in Japan [85], Korea [86], Taiwan [87], Malaysia [88, 89] and India [90]. However, these cases are highly doubtful. Given the possible fish hosts (salmonids) involved, it is unlikely that these cases involve *D. latum* infection. The fish hosts for *D. latum* are pike (*Esox lucius*), yellow perch (*Perca flavescens*), European perch (*Perca fluviatilis*), charr (*Salvelinus alpinus*) and burbot (*Lota lota*) from freshwater, and these fishes are seldom consumed in Asia. A case of *D. latum* infection reported in Saudi Arabia seems to have been acquired in Europe [91]. An alternative fish host for *D. latum*, redlip mullet (*Liza haematocheila*), exists in Korea [92], but there is a need to reconfirm the cestode species at the DNA level.

In contrast, predominant fish hosts for *D. nihonkaiense* are anadromous salmonids, *O. masou* and Pink salmon (*Oncorhynchus gorbuscha*)[43]. Chum salmon (*Oncorhynchus keta*) is also a fish host for *D. nihonkaiense* plerocercoids [93, 94]. Other marine diphyllobothriid species that resemble one another morphologically are also infective to humans: *D. yonagoense* [95], *D. cameroni* [96], *D. scoticum* [97], *D. hians* [98], *D. orcini* [99]and *D. pacificum* [100-102]. This makes the precise identification of these diphyllobothriid species more difficult. Moreover, with recent advances in international transport systems for foods including salmonids and trout, human diphyllobothriasis may occur in regions where it was previously unknown. Outside Japan, *D. nihonkaiense* infections confirmed by mitochondrial DNA analysis have recently been reported in France [93], Switzerland [94, 103] and New Zealand (Yamasaki et al., in preparation). A case of diphyllobothriasis caused by ingesting salmon imported from Canada was also reported, but the diphyllobothriid species was identified as *D. nihonkaiense* at the DNA level [104]. Clearly, molecular analysis will be necessary to prevent diagnostic confusion, such as that between *D. latum* and *D. nihonkaiense*, and for identification of diphyllobothriid species by DNA-based analysis to accumulate DNA information. In order to avoid the confusion that existed in Asia for a long period of time, more careful work should be done to assure the accurate diagnosis of diphyllobothriasis.

The fixation of tissues with formalin results in a well-preserved morphology, but it leads to a high degree of DNA and RNA fragmentation, which substantially constricts the spectrum of applicable molecular analysis [20]. In practice, it seems difficult to solubilize formalin-fixed tissues directly as compared with paraffin-embedded thin sections. In our experience, *cox1* gene fragments as long as 1.6-kb, 984-bp or 720-bp *cox1* can be amplified in some cysticercosis cases [12, 13]; however, smaller fragments can be stably amplified from formalin-fixed samples [6, 7, 9, 10]. The varying size of the PCR-products might be due to the quality of the formalin used in pathology and the time taken to fix samples.

As an alternative fixative, a versatile methacarn has recently been introduced for genomic DNA analysis in microdissected paraffin-embedded tissue specimens [105]. By using this fixative, extensive portions of DNA up to 2.8 kb can be amplified by nested PCR. On the other hand, a novel HOPE (Hepes-Glutamic acid buffer-mediated Organic solvent Protection Effect) fixative (DCS Innovative Diagnostik Systeme, Germany) is also currently utilized for PCR-based analysis [106] and *in situ* hybridization [107, 108], although the authors have not yet used it. The most remarkable feature of the HOPE fixative is the extremely low levels of nucleic acid degradation [109-111], making it possible to preserve and extract high molecular weight DNA and RNA of>20 kb and proteins in combination with excellent morphological results comparable to formalin-fixed tissues. In this study, the target genes were successfully amplified even from HE- and PAS-stained sections. This implies that stained specimens can be used for both routine and retrospective studies.

CONCLUSION

Mitochondrial DNA-based analysis using histopathological specimens from cestode zoonoses has become a powerful tool not only for routine and retrospective diagnosis, but also for the analysis of genetic polymorphism and species identification associated with pathogenicity. It is also indispensable in obtaining a definitive diagnosis, especially in cysticercosis cases where the histopathological findings are not confirmatory, as well as the accurate identification of diphyllobothriid tapeworms and taeniid cestodes.

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