MITOCHONDRIAL DNA DIAGNOSIS FOR CESTODE ZOONOSES: APPLICATION TO FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE SPECIMENS

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Abstract. A PCR-based technique was applied for definitive diagnosis using formalin-fixed, paraffin-embedded sections (FPES) from patients with cestode zoonoses, such as cysticercosis, alveolar echinococcosis, sparganosis, and diphyllobothriasis. DNA samples extracted from thin sections were used for PCR-amplification of mitochondrial cytochrome *c* oxidase subunit 1 gene. Although DNA were fragmented because of formalin fixation, smaller sizes of the gene fragments were stably amplified in all samples examined, and causative cestode species were identified by DNA sequencing. This study demonstrated that mitochondrial DNA analysis using FPES is a powerful tool, not only for routine and retrospective diagnosis, but also for genetic polymorphism analysis of the cestode species.

INTRODUCTION

Diagnosis of cestode zoonoses such as cysticercosis, echinococcosis, and sparganosis in humans is performed based on clinical manifestations, imaging examination, serology, and/or histopathology. Although the histopathology provides highly definitive evidences for diagnosis of cestodiasis, it is occasionally difficult to specify causative parasites due to the degeneration and/or calcification of the lesions, or artifacts in preparation. Recently, we reported two *Taenia solium* cysticercosis cases where histopathological findings were not confirmatory, but PCR-based mitochondrial DNA analysis using formalin-fixed paraffin-embedded sections (FPES) was highly useful for definitive diagnosis (Yamasaki *et al*, 2005, 2006a). Therefore, in order to diagnose such cases or to identify the cestode species found in the histopathological specimens, the authors have applied the PCR-based techniques for other parasitic diseases. In this study, the importance of molecular analysis using FPES in clinical diagnosis and parasitological studies was described with selected cestode zoonosis cases. In place of formalin, widely used as a tissues fixative, alternative fixatives that are currently used for molecular analysis are also introduced.

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MATERIALS AND METHODS

Formalin-fixed paraffin-embedded sections examined

Twenty samples from 19 patients with cysticercosis, alveolar echinococcosis (AE), sparganosis, and diphyllobothriasis were examined (Table 1). The tissues and plerocercoids resected from patients, and broad tapeworms expelled from diphyllobothriid patients were fixed with formalin and processed into FPES. Most FPES were prepared within the previous five years, but three samples from AE patients (cases 1-3) were archival specimens prepared 13-28 years ago. In sparganosis cases, formalin-fixed (case 1) and frozen plerocercoids (case 2) were used as DNA sources. For molecular analysis, unstained sections (5 sections with 10-μm thickness) were used; however, HE- (cases 2 and 3 in AE) and PAS-stained sections (case 6 in AE) attached onto the slide glasses were used.

DNA diagnosis

DNA samples were prepared from FPES using either DEXPAT (TaKaRa Shuzo, Japan) or DNA Isolator PS kit (Wako Pure Chemicals, Japan). The amplification of a target gene, a mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*), was performed with the protocol (94ºC, 1 minute; 58ºC, 30 seconds; 72ºC, 30 seconds; 35 cycles) (Yamasaki *et al*, 2004; 2005). The PCR-amplified products were run on 2-3% agarose gels or 10% polyacrylamide

gel; the nucleotide sequences of the amplicons were analyzed by direct DNA sequencing. The samples for sequencing were prepared using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (ABI PRISM, USA) and DNA sequencing was performed on an ABI PRISM 310 Genetic Analyzer.

RESULTS

Although the resected lesions and cestode parasites had been fixed with formalin prior to molecular analysis, the smaller sizes of *cox1* gene fragments were successfully amplified. Two different kits used for extraction of DNA from FPES provided similar results for amplification of target genes.

Cysticercosis

We have experienced seven cysticercosis cases confirmed by mitochondrial DNA analysis of the biopsied tissues. In Cases 1 and 3, the long sizes of 1.8-kb and 984-bp *cox1* were successfully amplified (Yamasaki *et al*, 2004, 2006a); however, smaller sizes (224-410 bp) of *cox1* fragments were stably amplified in other cases. In Case 4 with numerous cysts in the thigh and gluteal regions, for instance, resected cysts were extremely calcified and cysticercosis was not confirmed histopathologically (Fig 1-A). However, the 224-bp *cox1* fragments were amplified in FPES prepared from three cystic lesions (Fig 1-B, lanes 1-3), and it was revealed the lesions were derived from an Asian genotype of *T. solium* by DNA sequence analysis (Tsuda *et al*, 2007, in press). The patient was thought to have been exposed to *T. solium* approximately 60 years ago in Okinawa, where it was once an endemic area for *T. solium* cysticercosis. In Case 6 (Matsuoka *et al*, 2007), the patient presented with repeated epileptic seizures and was diagnosed as cysticercosis by histopathology (Fig 1-C). Because the patient had repeated travels during 1999-2005 to Korea, Bali (Indonesia), and Mexico where cysticercosis is still endemic, it was considered that the patient was exposed to *T. solium* in Mexico because of the patient's travel history in 2005. In order to presume the locality where the patient was infected, molecular analysis using FPES was performed. Subsequently, 224- and 720-bp *cox1*

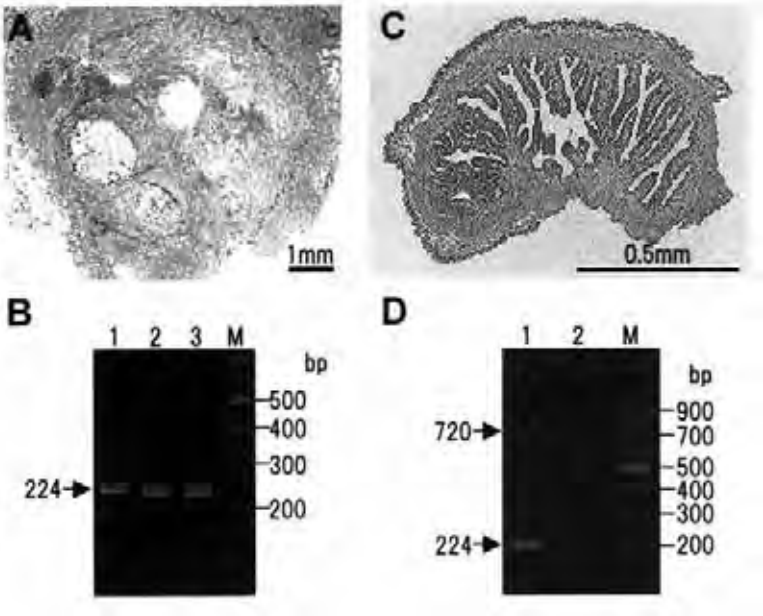

Fig 1- Cysticercosis. A and B, histopathological findings of biopsied lesion and 224-bp *cox1* gene fragments amplified from 3 cystic lesions (lanes 1-3) in case 4. C and D, a taeniid cysticercus showing characteristic labyrinth-like structure (from Matsuoka *et al*, 2007) and 224-bp and 720-bp (faint) *cox1* amplified by PCR (lanes 1 and 2) in case 6.

fragments were amplified by PCR (Fig 1-D), and the causative agent was identified as the Asian genotype of *T. solium* based on a nucleotide at position 723 of *cox1* (data not shown). With more detailed genetic polymorphism analysis of geographical isolates, it would be possible to specify the locality where the patient was exposed to *T. solium*.

Alveolar echinococcosis

In AE cases caused by *Echinococcus multilocularis* metacestodes, six cases were from Hokkaido, northern Japan, where AE is still endemic, and Cases 2 and 3 were from Rebun Island, a previously endemic area in Hokkaido. Case 1 was an interesting case from a remote area on Honshu, the main island of Japan, and was originally described as an autochthonous infection in 1979 (Yoshimura *et al*, 1979). According to Yoshimura *et al* (1979), the patient never went outside of Honshu and was diagnosed as AE by autopsy (Fig 3-A). Therefore, to confirm whether *E. multilocularis* from Case 1 is identical to *E. multilocularis* isolates from Hokkaido, PCR-based sequence analysis was performed using archival FPES samples. As a result, only smaller sizes (99-123 bp) of *cox1* fragments were amplified (Fig 2-B). The 220-bp *cox1* was not amplified, even in nested PCR (data not shown). For determining the complete sequence of *cox1* from Case 1, overlapped *cox1* fragments with molecular sizes of 100~131-bp were amplified by PCR using 16 primer sets (data not shown). Subsequently, it was proven *E. multilocularis cox1* from the Case 1 was identical to those of the isolates from Hokkaido. It is still undetermined how the patient got infected with *E. multilocularis* in the remote area.

Conversely, in AE Cases 2, 3, and 6, HE-(Cases 2 and 3) and PAS-stained sections (Case 6) attached onto the slide-glasses were used as DNA sources. In these cases, DNA Isolator PS kit was utilized. In Cases 2 and 3, 220-bp *cox1* fragments were amplified (data not shown). In Case 6, in addition to 220-bp *cox1*, 410-bp *cox1* fragment was amplified (Fig 2-D, lanes 1-2), however the amplification of longer 825-bp *cox1* fragment was not successful (Fig 2-D, lane 3). These results demonstrate that HE- and PAS-stained sections are also useful materials for retrospective studies

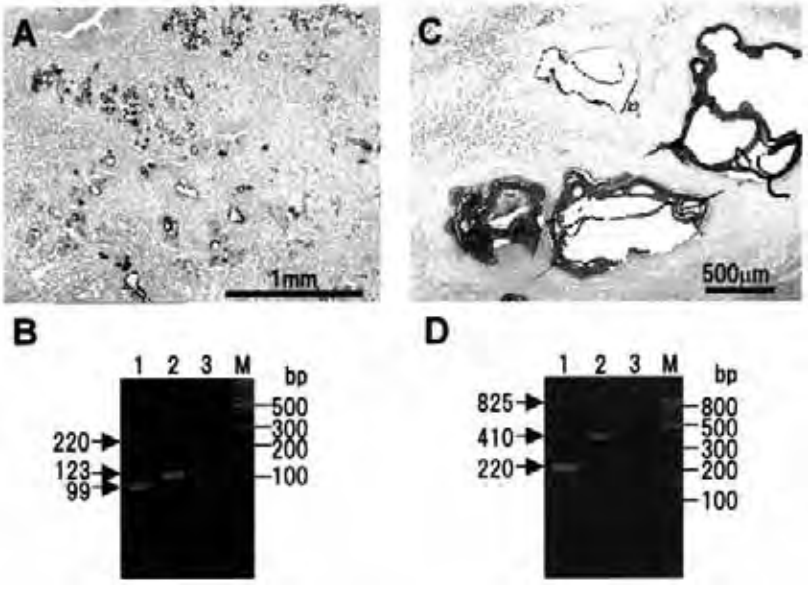

Fig 2- Alveolar echinococcosis. A and B, typical histological finding showing AE (PAS stain) and smaller sizes of *cox1* amplified by PCR from the archival FPES (lanes 1 and 2) in case 1. C and D, PAS-stained histopathology and PCR-amplified 224- and 410-bp *cox1* fragments (lanes 1 and 2) in case 6. No PCR products with molecular sizes of 220- and 825-bp *cox1* fragments were amplified in case 1 (lane 3 in B) and case 6 (lane 3 in D), respectively.

because many histopathological specimens are stained with HE and/or PAS.

Sparganosis and diphyllobothriasis

Sparganosis caused by the infection of larval *Spirometra* plerocercoid is sporadically found in Japan. The diagnosis is easy if the plerocercoid is surgically removed from patients presenting creeping eruption. In Japan, the *Spirometra* species causing sparganosis is considered *Spirometra erinaceieuropaei*. However, whether the causative plerocercoid is really *S. erinaceieuropaei* is difficult to determine based on the morphology. To identify the plerocercoid species accurately or examine genetic polymorphism among the plerocercoids, molecular analysis was performed. Fig 3-A shows a formalin-fixed plerocercoid used for DNA extraction from Case 1(Yoshikawa *et al*, 2002). The target *cox1* with molecular sizes of 445~480-bp *cox1* fragments were successfully amplified from the plerocercoid (lanes 1 and 2 in Fig 3-B, Case 1) as well as other two cases, and sequence analysis of the PCR products revealed the *Spirometra* plerocercoids were all identified as *S. erinaceieuropaei*, although a genetic divergence (1.1-2.4%) was observed.

Diphyllobothriasis cases caused by the infection of the adult broad tapeworm, *Diphyllobothrium* spp, are frequently found in regions where people eat raw salmon and trout that cross the oceans. *Diphyllobothrium latum* that is distributed in North Europe has been considered a cestode species causing diphyllobothriasis in humans for over a century. However, the species was described as *Diphyllobothrium nihonkaiense*, based on the morphology (Yamane *et al*, 1986), and it has been demonstrated that *D. nihonkaiense* is a distinct species from *D. latum* at the mitochondrial genome levels (Nakao *et al*, 2007). *D. nihonkaiense* is currently recognized as a cestode species causing diphyllobothriasis in Japan. According to Yamane *et al* (1986), one distinct morphology for differentiating *D. nihonkaiense* from *D. latum* is the position of cirrus sac: it is situated obliquely against the anterior-posterior axis in *D. nihonkaiense*, whereas it is situated horizontally in *D. latum*. However, the morphological criterion is not absolute for the differentiation of the species because the position of segments to be examined or preparation of sagittal sections. We also rarely find diphyllobothriasis caused by the marine *Diphyllobothrium* cestodes, other than

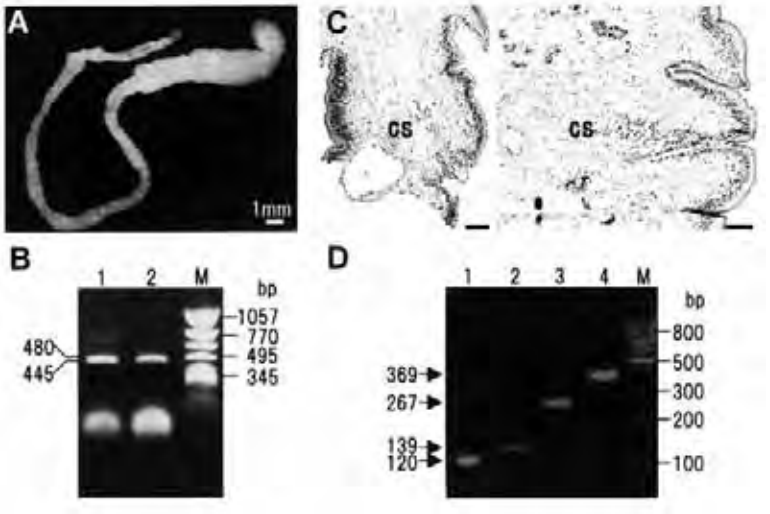

Fig 3- Sparganosis and diphyllobothriasis. A and B, a formalin-fixed plerocercoid used as DNA source (from Yoshikawa *et al*, 2003) and PCR-amplified 445-480-bp *cox1* fragments (lanes 1-2) in case 1. C, sagittal sections showing different positions of cirrus sac (cs) of 2 diphyllobothriid tapeworms expelled from case 1 (left) and case 2 (right). D, PCR-amplified 120-369-bp *cox1* fragments (lanes 1-4) in case 1. Bars in C = 100 μm.

D. nihonkaiense, and it may be necessary to identify the cestode species at molecular level in parasitology. Fig 3-C shows sagittal sections of diphyllobothriid cestodes expelled from two Japanese children (Cases 1 and 2). The cestode (left) can be identified as *D. nihonkaiense* from the cirrus sac situated obliquely, but the other one (right) shows morphology resembling *D. latum*. In molecular analysis using FPES of the segments, 120~396-bp *cox1* fragments were amplified in a diphyllobothriid tapeworm (Fig 3-D, lanes 1-4 from Case 1) as well as other two tapeworms, and all diphyllobothriid tapeworms were identified as *D. nihonkaiense*.

DISCUSSION

In the present paper, the authors stressed the importance of molecular-pathological diagnosis using FPES in clinical diagnosis and parasitology with selected cestode zoonosis cases. In echinococcosis and sparganosis and diphyllobothriasis, because serology and histopathology and/or morphology provide reliable diagnostic results, molecular diagnosis may be not necessary for clinical diagnosis. However, the serological and histopathological diagnosis of cysticercosis is occasionally not confirmatory. Cysticercosis cases with a solitary cyst and calcified lesions are frequently seronegative, although the cysticercosis with multiple cysts (Case 5, Table 1) is serologically positive as pointed by Sako *et al* (2000) and Sako and Ito (2001). Histopathological findings are also not absolute in cases where the cystic lesions are degenerated and/or calcified (Yamasaki *et al*, 2004, 2005; Tsuda *et al*, 2007; Ishikawa *et al*, 2007). Furthermore, the causative *Taenia* species is exclusively *T. solium* in cysticercosis in humans; however, there have been reported cysticercosis cases caused by zoonotic *Taenia* species other than *T. solium*, such as *Taenia crassiceps* in AIDS patients or by unusual racemose-type *T. solium* in HIV-positive individuals (reviewed by Yamasaki *et a*l, 2006b, c). Having considered these situations, molecular analysis using FPES is highly informative and useful, not only for identification of the *Taenia* species in cysticercosis (Yamasaki *et al*, 2004, 2005, 2006b, c; Ito *et al*, 2006), but also for genetic polymorphism analysis of causative cestode species causing echinococcosis, sparganosis and diphyllobothriasis.

Although it is known that fixation in formalin of tissues and processing of tissues to paraffin wax degrades DNA molecules because of cross-linking caused by formalin, it is possible to extract DNA of up to 200 bp from the archival FPES (Bianchi *et al*, 1991). In practice, it seems to be difficult to solubilize formalin-fixed tissues directly as compared with thin sections. In our experience, 1.8-kb, 984-bp or 720-bp *cox1* were able to amplify in some cysticercosis cases (Yamasaki *et al*, 2006a; Matsuoka *et al*, 2007); however, smaller 224-bp *cox1* fragments are more stably amplified in most cysticercosis cases (Yamasaki *et al*, 2005, 2006a; Matsuoka *et al*, 2007; Tsuda *et al*, 2007; Ishikawa *et al*, 2007). In AE cases, only 100-131-bp *cox1* fragments were amplified in the archival specimen as shown in Fig 2. In recently prepared specimens, 220-410-bp *cox1* fragments were amplified; however, the amplification of longer 825 bp-*cox1* fragment was not successful. In sparganosis and diphyllobothriasis cases, 445-480-bp and 120-396-bp *cox1* fragments were amplified, respectively, from FPES although amplification of larger sizes of *cox1* fragments were not performed in these zoonosis cases. The sizes of the PCR products could be due to the quality of formalin used in pathology departments and the times taken to fix samples.

In this study, the target genes were successfully amplified when HE- and PAS-stained sections from AE patient were used. This implies that stained specimens are available for routine and retrospective studies. The PCR-based diagnosis was also useful for differentiating AE from disease similar to AE having tortuous laminated layers (Yamasaki *et al*, unpublished data). In our department, although the FPES is now used for diagnosing parasitic diseases and genotyping of the parasites, it is highly interested in demonstrating parasite-specific DNA by in situ PCR using FPES, in particular, in the obsolete cysticercosis case where the structure of cysticercus is extremely degenerated. The PCR-based diagnosis using FPES is highly beneficial for providing definitive information, not only

Table 1
Demographic data on cestode zoonosis cases examined in this study.

Case #	Patient	Lesion	Serology	Histopathology	Year	Reference
Cysticercosis						
1	53/F, Japanese	Brain (solitary)	(-)	Confirmed	2002	Yamasaki *et al*, 2004
2	9/F, Filipino	Brain (solitary)	(-)	Not confirmed	2003	Yamasaki *et al*, 2005
3	83/M, Japanese	Muscles (calcified)	(-)	Not confirmed	2003	Yamasaki *et al*, 2006
4	87/M, Japanese	Muscles (calcified)	(-)	Not confirmed	2005	Tsuda *et al*, 2007
5	28/F, Indian	Brain (multiple)	(+)	Not confirmed	2005	This study
6	24/F, Japanese	Brain (solitary)	(-)	Confirmed	2005	Matsuoka *et al*, 2007
7	38/F, Japanese	Brain (solitary)	(-)	Not confirmed	2005	Ishikawa *et al*, 2007
Alveolar echinococcosis						
1	62/F, Japanese	Liver	Not done	Confirmed	1978	Yoshimura *et al*, 1979
2	73/F, Japanese	Liver	Not done	Confirmed	1982	This study
3	62/F, Japanese	Liver	Not done	Confirmed	1992	This study
4	?/F, Japanese	Liver	Not done	Confirmed	1993	This study
5	48/F, Japanese	Liver, bone	(+)	Confirmed	2004	This study
6	64/F, Japanese	Liver	(+)	Confirmed	2006	This study
7	47/F, Japanese	Liver	(+)	Confirmed	2006	This study
Sparganosis						
1	64/M, Japanese	Brain	(+)	Confirmed	2002	Yoshikawa *et al*, 2002
2	67/F, Japanese	Abdominal skin	(+)	Confirmed	2005	This study
3	58/F, Japanese	Orbit, forehead	(+)	Confirmed	2006	This study
Diphyllobothriasis						
1	4/M, Japanese	Intestine	Not done	Confirmed	2006	This study
2	3/F, Japanese	Intestine	Not done	Confirmed	2006	This study

in clinical parasitology, but also for genetic polymorphism analysis among the causative cestode species. Such technologies are currently applied for various diseases, for instance, parasitic diseases (Muller *et al*, 2003; Yamasaki *et al*, 2004, 2005, 2006a; Boer *et al*, 2006; Rivasi *et al*, 2006), tuberculosis (Hofman *et al*, 2003; Schewe *et al*, 2005), Lyme borreliosis (Chou *et al*, 2006), virus-related diseases (Ikegaya *et al*, 2005; Bryan *et al*, 2006) and cancers (Paik *et al*, 2005; Yatabe *et al*, 2006).

Fixation of tissues with formalin results in well-preserved morphology, but it leads to fragmentation of DNA and RNA to a high degree, which substantially constricts the spectrum of applicable molecular analysis (Bianchi *et al*, 1991; Vollmer *et al*, 2006). As an alternative fixative, a versatile methacarn has been introduced for genomic DNA analysis in microdissected paraffin-embedded tissue specimens (Uneyama *et al*, 2002). By using the fixative, extensive portions of DNA of up to 2.8 kb could be amplified by nested PCR using DNA extracted from a 1 x 1-mm area of cerebral tissues. On the other hand, a novel HOPE (Hepes-Glutamic acid buffer-mediated Organic solvent Protection Effect)-fixative (DCS Innovative Diagnostik Systeme, Germany) has also been currently utilized for PCR-based analysis (Goldmann *et al*, 2002; Sen Gupta *et al*, 2003), *in situ* hybridization (Umland *et al*, 2003; Vollmer *et al*, 2006), immunohistochemistry (Olert *et al*, 2001; Umland *et al*, 2003; Vincek *et al*, 2003), Western blot (Uhlig *et al*, 2004) and tissue microarray analysis (Goldmann *et al*, 2004, 2005; Vollmer *et al*, 2006). The most remarkable feature of the HOPE-fixative is the extremely low degradation of nucleic acids (Olert *et al*,

2001; Wiedorn *et al*, 2002; Vincek *et al*, 2003); it allows us 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 AE, it would be highly interesting to investigate gene-expression profiling between echinococcal foci and non-echinococcal tissues using a DNA microarray prepared from paraffin-embedded sections fixed with HOPE.

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