MOLECULAR IDENTIFICATION OF *TAENIA SOLIUM* CYSTICERCUS GENOTYPE IN THE HISTOPATHOLOGICAL SPECIMENS

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Abstract. PCR-based molecular diagnosis was done for identification of causative agents found in paraffin-embedded specimens that were resected from two suspected neurocysticercosis patients. DNA samples were extracted from tissues or sections and cytochrome *c* oxidase subunit 1 gene and cytochrome *b* gene were amplified for the detection of taeniid DNA. Two different genes were successfully amplified in both specimens, but the sizes of amplified products seemed to depend on the quality of DNA. Based on the nucleotide sequences of the PCR-amplified genes, the causative agents from two cases were identified as *T. solium* Asian genotype. When infection with *T. solium* is not confirmed by histopathological examination, molecular diagnosis will be more useful for definitive diagnosis.

INTRODUCTION

Cysticercosis is a zoonosis caused by infection with larval *Taenia solium* cysticercius. When cysticercus develops in the central nervous system, severe neurocysticercosis (NCC), characterized by epileptic seizures, may occur. The disease has been recognized as an emerging and re-emerging disease in both developed and developing countries (Schantz *et al,* 1998), and approximately 20 cases of cysticercosis, most of which are imported cases, have been reported in last decade in Japan (Yamasaki *et al* 2004a). For the diagnosis of the disease, imaging diagnosis using computed tomography (CT) and magnetic resonance imaging (MRI) and serological examination have been routinely performed (Tsang *et al,* 1989; Wilson *et al*, 1991; Del Brutto *et al*, 1998). If postoperative specimens are available, it is also useful to confirm the causative agent histopathologically. However, it is not always possible to identify the causative agent due to suboptimal preparation of the sections, the degree of calcification of the lesion, or incomplete formation of the hooklets (Margono *et al*, 2003). Recently, the causative agent found in the postoperative specimen from a suspected NCC patient was identified based on molecular diagnosis (Yamasaki *et al*, 2004b). In this paper, PCR-based molecular diagnosis was introduced for the identification of causative agents in histopathological specimens prepared from suspected NCC patients.

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MATERIALS AND METHODS

Histopathological specimens

Two postoperative specimens were obtained from the following patients: Case 1 was well documented (Yamasaki *et al*, 2004b). Briefly, the patient was a 53-year-old Japanese woman who developed aphasia and numbness of the right arm and NCC was highly suspected by CT and MRI findings. In order to differentiate from a cerebral tumor, craniotomy was performed. The resected lesion was fixed with formalin and then processed for histopathological examination. As shown in Fig 1-A, a taeniid cysticercus characterized by suckers and spiral canals was observed, but no hooklets were confirmed in any sections. The second sample was from a 9-year-old girl who was born in the Philippines and grew up in Japan. She developed convulsions and a solitary mass lesion was found in the left parietal lobe by CT scan. The lesion was surgically removed to rule out a cerebral tumor and processed for histopathological observation. A characteristic structure of taeniid cysticercus was not observed due to the denaturation of the tissue (Fig 1-B). No antibody against *T. solium* antigen was detected in these two cases. Therefore, in order to confirm whether the causative agents in these two cases were really *T. solium*, DNA diagnosis was performed using paraffin-embedded specimens prepared from these two patients.

DNA diagnosis

DNA samples from paraffin-embedded specimens were prepared as follows: a paraffin-block (case 1) or thin-sections (case 2) were heated at 70°C and tissues were separated from paraffin. The separated tissues were then lysed in 50~60 μl of 0.02 N sodium

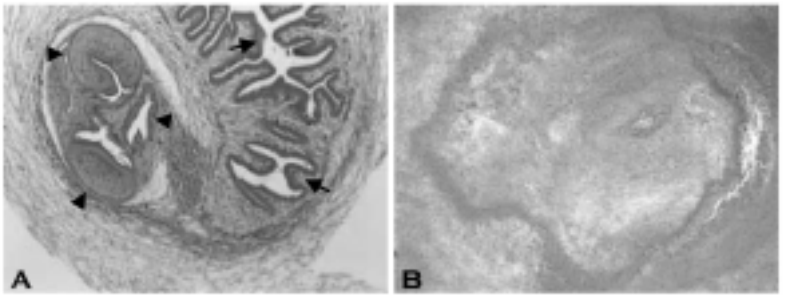

Fig 1- Histopathological examination of the lesions. The sections were stained with hematoxylin and eosin. A, case 1. The invaginated scolex with suckers (arrowheads) and spiral canal (arrow) are observed but no hooklet was seen. Original magnification was 25 x. B, case 2. The characteristic structure was lost due to the denaturation of the tissue. Original magnification was 40 x.

Table 1
Primers used for the amplification of gene fragments from taeniid parasites and human.

Gene	Primer sequence	Positions	Remarks
cox1	F0: 5´-GTTAATGTTGACTAGATGTTTTCA-3´		tRNA gene for tryptophan
	F1: 5´-TTGTTATAAATTTTTGATTACTAAC-3´	165-189	specific primer for *T. solium* Asian genotype
	F2: 5´-TTTATGACTAATATATTTTCTCGTAC-3´	517-542	
	F3: 5´-TATTTGATCGTAAATTTAGTTCTGCGTT-3´	629-656	
	F4: 5´-GGTAGATTTTTTAATGTTTTCTTTA-3´	429-453	specific primer for *T. solium* American/African genotype
	F6: 5´-TACTGTGGGTTCTTTTATATCTGCTTTTAG-3´	1365-1394	
	R3: 5´-GATGACCAAAAAATCAAAACATATGTTG-3´	721-694	
	R4: 5´-ATTATCATAGTAACAGAACTAAAAAATAC-3´	935-907	
	R5: 5´-CATTTTCATTATGTTATGTCATTAGGGTC-3´	1129-1157	
	R6: 5´-ACAGGACTCATAAAAAAATTCACCAAACA-3´	1502-1474	
	R7: 5´-TCCACTAAGCATAATGCAAAAGGC-3´		ribosomal RNA large subunit gene
cob	F2: 5´-GTCAAAAGATTCTTTTTTTACTTGGT-3´	180-205	
	F3: 5´-GTGTTAAAGTGTTGTTTATCTTACTT-3´	230-255	
	F4: 5´-TTATTGGGCTGCTACTGTTTTGACAT-3´	390-415	
	R2: 5´-CCCTTCTTTCTATAACTTGAATAAT-3´	305-281	
	R3: 5´-TATAGATGTCAAAACAGTAGCAGCC-3´	420-396	
	R4: 5´-TGTCAACTATCAATCCCAGAATGAT-3´	560-535	
TH01 locus	TH01F: 5´-ATTCAAAGGGTATCTGGGCTCTGG-3´		short tandem repeat locus of human tyrosine hydroxylase gene
	TH01R: 5´-ATAATCGGGAGCTTTTCAGCCCAC-3´		

hydroxide containing 10~20 µl of proteinase K at 90° C for 30~60 minutes. After removal of proteinase K using phenol/chloroform, DNA samples were purified and used directly for PCR as template DNA. The taeniid and human DNA samples as controls were prepared from *T. solium* Asian genotype and human peripheral blood using DNeasy Tissue Kit (Qiagen) (Nakao *et al,* 2002; Yamasaki *et al,* 2002, 2004c) and QIAamp DNA Blood Mini Kit (Qiagen), respectively. For the amplification of taeniid genes, cytochrome *c* oxidase subunit 1 gene (*cox1*) and/or cytochrome *b* gene (*cob*) were targeted. For the evidence of human DNA, the short tandem repeat locus of tyrosine hydroxylase gene (TH01 locus) was amplified (Edwards *et al*, 1991; Polymeropoulos *et al*, 1991). All primers used for the PCR-amplification are listed in Table 1. The samples for DNA sequencing were prepared using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit and DNA sequencing was performed on an ABI PRISM 310 Genetic Analyzer.

RESULTS

In case 1, approximately 1.8 kb- and 984 bp-*cox1* fragments were successfully amplified (lanes 1 and 3 in panel A, Fig 2) as well as the *T. solium* Asian genotype as control (lane 4). Nevertheless the specimen had been fixed with formalin and mitochondrial DNA did not seem to be fragmented. In contrast, such *cox1* fragments were not amplified in case 2 (lanes 6 and 7 in panel B, Fig 2) compared with those of *T. solium* Asian genotype as control (lanes 13 and 14). However, less than 400 bp-*cox1* fragments were successfully amplified (lanes 1-5, panel B) as well as intact *T. solium* (lanes 8-12). As in the case of *cob*, less than 300 bp-*cob* fragments were successfully amplified, although the amplification of ~600 bp-fragment was not succeeded (data not shown), suggesting that the DNA was fragmented due to the use of formalin in case 2. Although no trial was done to amplify TH01 locus in case 1, the locus was also detected in case 2 (lane 1 in the panel C, Fig 2).

Within *cox1* consisting of 1,620 bp, nucleotides at 11 positions served as differential markers for human taeniid cestodes. Fig 3 shows the representative three nucleotides, based on the sequence data of the PCR-amplified products in cases 1 and 2, together with corresponding regions of *cox1* from known taeniid parasites.

DISCUSSION

A nucleotide at position 672 is for differentiation of taeniid species, and the nucleotides at positions 690 and 723 are pertinent for differentiation of Asian and American/African genotypes of *T. solium* (Yamasaki *et al*, 2002, 2004b). The causative agents from both patients had adenine, guanine and cytosine at positions 672, 690 and 723, respectively, and were identified as *T. solium* Asian genotype. In case 2, the nucleotide sequence of *cob* also supported that the causative agent was *T. solium* Asian genotype (data not shown).

As demonstrated in the present study, if post-operative specimens are available, but morphological findings are not confirmatory, molecular diagnosis would be more useful for the definitive diagnosis of NCC. To date, the only taeniid species that causes cysticercosis in humans exclusively is *T. solium,* including racemose-type cysticercus (*Cysticercus racemosus*). However, there could be cysticercosis caused by other taeniid species of zoonotic origins other than *T. solium*. From this point of view, molecular diagnosis will be significant. For the molecular identification of causative agents, it is strongly recommended to preserve materials in >80% ethanol as much as possible, but formalin-fixed and paraffin-embedded specimens are also usable for DNA analysis.

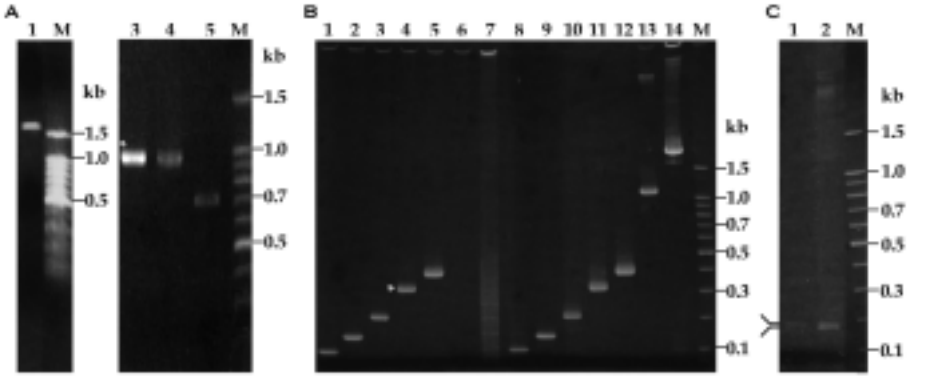

Fig 2- Cytochrome *c* oxidase subunit 1 gene fragments amplified by PCR. Panel A, case 1. Lanes 1 and 3, products amplified using F1/R7 and F1/R5 primers, respectively; lanes 4 and 5, *cox1* from *T. solium* Asian and *T. solium* American/African genotypes amplified using *T. solium* genotype-specific primers (F1/R5 and F4/R5). PCR products were electrophoresed on a 1% agarose gel at 100V. Panel B, case 2. Lanes 1-7, *cox1* amplified using different primer sets of F3/R3, F6/R6, F2/R3, F3/R4, F5/R6, F1/R5, and F0/R7 from the left lanes. Lanes 8-14, *cox1* from *T. solium* Asian genotype using same primer sets. Panel C, detection of TH01 locus. Lane 1, case 2; lane 2, human volunteer as control. Trimeric and tetrameric repeats are shown. PCR protocol for *cox1* was performed according to Yamasaki *et al* (2002, 2004b). PCR protocol for TH01 locus consisted of 30 cycles of 94°C for 60 seconds, 60°C for 30 seconds and 72°C for 30 seconds (Edward *et al*, 1991; Polymeropoulos *et al*, 1991). PCR products in panels B and C were run on 4~10% gradient polyacrylamide gels at 140V and stained with ethidium bromide.

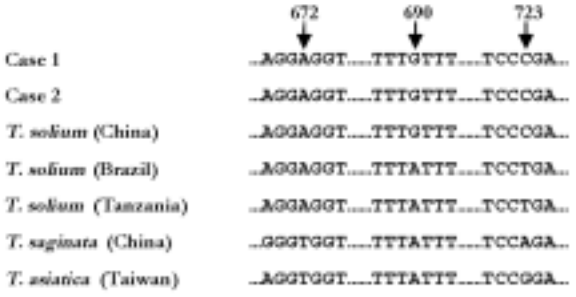

Fig 3- Alignment of the partial nucleotide sequences of cytochrome *c* oxidase subunit 1 gene. *T. solium*-specific and *T. solium* genotype specific nucleotides are shown by arrows. The numbers indicates the nucleotide positions in the 1,620-bp *cox1* gene. The nucleotide sequences of the causative agents in the cases 1 and 2 are aligned with those from the Asian genotype of *T. solium* (AB066485), the American/African genotype of *T. solium* (AB066492 and AB066493), *Taenia saginata* (AB066495) and *Taenia asiatica* (AB066494).

ACKNOWLEDGEMENTS

The authors thank S Matsunaga (Department of Neurosurgery, National Hospital, Yokohama Medical Center, Yokohama, Japan), K Nagakura and K Suzuki (Department of Infectious Diseases and Department of Pediatrics, Tokai University School of Medicine, Isehara, Japan) for kindly providing histopathological specimens, and Y Itoh (Department of Forensic Medicine, Juntendo University School of Medicine, Tokyo, Japan) for providing primer sets for the amplification of human gene. This work was supported in part by a grant-in-aid for scientific research from the Japan Society for Promotion of Science to AI (14256001).

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