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Anomalous segmentation of *Diphyllobothrium nihonkaiense*

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Nucleotide sequences determined in this report were deposited into DDBJ/EMBL/GenBank databases under the accession numbers of AB512013 (18S rDNA), AB508837 (*cob*) and AB508838 (*cox1*).

Abstract

An anomalous tapeworm with abnormal segmentation was obtained from 6-year-old boy in Japan. The tapeworm consisted of proglottids with slanted anterior and posterior margins of proglottids and 4-6 sets of reproductive organs arranged between the margins. The morphology of the tapeworm did not correspond to any of the described cestodes. However, molecular identification based on nuclear and mitochondrial genes clearly showed the tapeworm was *Diphyllobothrium nihonkaiense*.

Key words:

Diphyllobothrium nihonkaiense

Anomalous segmentation

Molecular identification

Human diphyllobothriasis spreading worldwide, is a cestode zoonosis caused by eating raw or undercooked fish infected with larvae of *Diphyllobothrium* spp. (Diphyllobothriidae)[1, 2]. There are more than 10 species known to cause human infection [1, 3]. The main pathogenic organism is *Diphyllobothrium nihonkaiense* in Japan [4, 5], while *Diphyllobothrium latum* is common in European countries [6]. *D. nihonkaiense* had been considered as the same species with *D. latum* until 1986, when Yamane *et al.* [7] differentiated the both species. Although those tapeworms are morphologically similar to each other, their second intermediate hosts are crucially different. *D. nihonkaiense* exploits anadromous Pacific salmons such as *Oncorhynchus masou masou* (masu salmon), *Oncorhynchus gorbuscha* (pink salmon) and *Oncorhynchus keta* (chum salmon), whereas *D. latum* exploits freshwater fish such as *Perca fluviatilis* (perch) and *Esox lucius* (pike). Another tapeworm *Diplogonoporus balaenopterae* belonging to the family Diphyllobothriidae also causes intestinal infections in Japan [8, 9]. The most crucial morphological difference between the genera *Diphyllobothrium* and *Diplogonoporus* is the number of genitalia per segment. Except for the abnormal development of the strobila, *Diphyllobothrium* spp. have a single set of genitalia, whereas *Diplogonoporus* spp. have double sets.

Species identification of these tapeworms has been basically based on the morphology of larval and adult stages. However, the morphological

identification is little reliable, particularly in sibling species and atypical individuals. Therefore, molecular diagnostic tools using mitochondrial DNA (mtDNA) and nuclear DNA markers have been widely utilized for the identification of tapeworms [10-16]. Here we report a clinical case infected with an abnormal tapeworm, which was later identified as *D. nihonkaiense* by molecular diagnosis.

A 6-year-old boy, living in Oyama City, Tochigi Prefecture, shed a tapeworm for the first time on January 2008, and was referred to Jichi Medical University Hospital on September 3 but without specimen. No treatment was done at that time. Next time, he came on October 24, 2008 with a newly expelled worm specimen with 40cm of length. He had not been claiming any subjective symptom other than releasing worms. Three weeks later, the patient was treated with praziquantel and laxative. We examined the patient's feces for 2 days and the tapeworm was only found in the feces obtained 24 hours after the treatment. Because the tapeworm was considerably damaged and fragmented in much feces, the scolex part was unfortunately not found after all. Although the scolex was not found after the treatment, the whole tapeworm was considered to be released because the obtained mass of proglottids contained a narrow part close to the neck region.

The tapeworm consisted of only abnormal proglottids throughout the obtained strobila (Fig. 1A). Eggs found in the feces of the patient resembled

the eggs of *Diphyllobothrium*. The worm obtained in October was fixed with 70% ethanol, and morphological observation and molecular diagnosis were conducted at Asahikawa Medical College. A part of the strobila was whole-mounted and stained with Semichon's acetic carmine. Some parts were embedded in paraffin, cut into 5 µm and stained with hematoxylin and eosin. Another part was refixed with 2.5% glutaraldehyde and used for scanning electron microscope (SEM) observation.

The width of the strobila was about 1 cm, and a maximum thickness was 650 µm (Fig. 1A, B). Margins of proglottids slanted to left both on the ventral and dorsal side. From the frontal view, 4-6 sets of reproductive organs were arranged between the anterior and posterior margins of proglottids (Fig. 1A, C). Some of the uteri contained eggs. SEM observation revealed the genital pores opened only on the ventral surface (data not shown). The eggs had an operculum at one end and an apical knob at the other. The size of the eggs (n = 20) were 67.6 (61.8 – 73.8) × 45.1 (42.4 – 48.1) µm (Fig. 1D).

The parasite DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen), and the nuclear DNA fragment of 18S ribosomal RNA gene (18S rDNA) was amplified by polymerase chain reaction (PCR) using the universal eukaryotic primers ERIB1 (5'-acctggttgatcctgccag-3') and ERIB10 (5'-cttccgcaggttcacctacgg-3')[17]. The mtDNA fragments of cytochrome *c* oxidase subunit I (*cox1*) and cytochrome *b* (*cob*) were also

amplified with two sets of primers; Diphyllo-Cox1-F/R (5'-tagactaagtgttttcaaaacacta-3'/5'-atagcatgatgcaaaagg-3') and Diphyllo-Cob-F/R (5'-tgataggttatttaaactggc-3'/5'-tcaacagttgaaacaacca). All PCRs were performed in 20 μL volumes containing 0.5 units of Ex Taq (Hot Start Version (TaKaRa), 0.2 mM of dNTP, 1 × Ex Taq Buffer with a final $MgCl_2$ concentration of 2.0 mM, 15 pmol of each primer and 1.0 μL of genomic DNA. Main thermal reactions were performed as follows; 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60s (*cox1* and *cob*), and 35 cycles of 95°C for 1 min, 54°C for 1 min and 72°C for 2 min (18S rDNA). A BigDye™ Terminator v1.1 and a 310 DNA sequencer (Applied Biosystems) were used for the direct sequencing of the PCR products.

The partial sequence of 18S rDNA (2134 bp) and the complete sequences of *cob* (1107 bp) and *cox1* (1566 bp) were determined and compared with available sequences in the GenBank database. The 18S rDNA sequence was completely identical with that of *D. nihonkaiense* (AB374225). Both *cob* and *cox1* sequence showed more than 99.5% identities with those of *D. nihonkaiense* (EF420138), whereas the similarities of the *cox1* sequence with *D. dendriticum* (AM412738) and *D. latum* (FM209181) were 93.2% and 92.6%, respectively.

Morphologically, the most notable feature of the strange worm was the slanted anterior and posterior margins of proglottids. Because the margins of proglottids on the ventral and dorsal tegument were not parallel to each

other, the tapeworm was considered not to have “segments” in a usual meaning. Another feature of the tapeworm was multiple sets of reproductive organs arranged between the anterior and posterior margins of proglottids. Although the worm was thought to be a member of the genus *Diphyllobothrium* or *Diplogonoporus* because of the egg morphology, there was no described species having such a strange strobila. While morphological observation did not identify the species, 18S rDNA, *cox1* and *cob* sequences of the parasite showed high similarity to that of *D. nihonkaiense*. 18S rDNA is rather conservative and unsuitable for interspecific characterization among some *Diphyllobothrium* species such as *D. latum, D. dendriticum* and *D. ditremum* [13, 14]. On the other hand, *cox1* is more variable and can clearly discriminate those three species [13, 14]. Intraspecific mtDNA sequence divergence in *D. nihonkaiense* is relatively high, around 2% in *cox1* and NADH dehydrogenase subunit 3 (*nad3*) [10, 11]. The complete *cox1* sequence of the tapeworm obtained in the present study showed 99.7% similarity with *D. nihonkaiense*. Thus, molecular analysis clearly showed that the strange tapeworm was an anomalous *D. nihonkaiense*. Occurrence of abnormal progrottids of *D. nihonkaiense* was intensively examined with 140 specimens preserved in 17 universities and research institutes in Japan, and many types of abnormal forms were recorded [18]. They were divided into 12 major types including that with 2-4 sets of genital organs per segments and/or abnormal

segmentation. Koga and Iwata [18] found that the occurrence of abnormal forms of *D. nihonkaiense* was rather common. However, none of the abnormal types in the previous report was similar to the present case. Most of the cases in the previous report consisted of both normal and abnormal segments, whereas the specimen in the present case was composed of only abnormal forms throughout the obtained strobila. The cause and the mechanism of the occurrence of the abnormal proglottids in the diphyllobothriid cestodes are still unknown. Koga and Iwata [18] speculated that the abnormal forms, such as double sets of genitalia in one segment, show the progress of subdivision of the mature progrottids. On the other hand, Anderson [19] obtained abnormally large and slender plerocercoids of *Diphyllobothrium dendriticum* from rainbow trout *Oncorhynchus mykiss* experimentally infected by the intraperitoneal injection of plerocercoids with scolex removed. The author suggested the scolex or neck region control the growth and development of cestodes and the abnormal development would occur if those parts were removed. The abnormal segmentation in the present case was considered to have occurred not among the mature progrottids but in the scolex or neck region resulting from the failure of the regulation of developmental gene expression in early morphogenetic process, because the abnormal form appeared constantly in the specimen.

Retrospective interview with the patient could not find any history of

eating raw or undercooked salmons, except for a grilled rainbow trout farmed in the river in Japan. Rainbow trout has not been reported as the host of *D. nihonkaiense* so far. Besides, extensive fieldwork on masu salmon, *O. masou masou*, in Hokkaido, the north island of Japan, revealed that the juveniles in the river before migrating to the sea were not infected with *D. nihonkaiense*, whereas the adults returned to the rivers had prevalences ranging from 0 to 50%. It strongly suggests that *O. masou masou* acquires the parasite during their migration through the ocean [20]. It is, therefore, highly unlikely that the grilled rainbow trout was the source of infection. The origin of the strange *D. nihonkaiense* remains unknown. Until recently, *D. nihonkaiense* was considered to distribute only around Japan and Far East Russia. However, clinical cases of *D. nihonkaiense* have been reported in France, Switzerland, Canada and New Zealand in the past 5 years [12, 14, 15, 21, 22]. The authors of these reports suspected that the cases were caused by eating Pacific salmons imported from Pacific coast of North America, except for the case of a Czech tourist who ate 5 species of Pacific salmons in Canada [14]. These case reports indicate the expansion of *D. nihonkaiense* infection resulted from the worldwide transportation of fresh salmons, and a broader geographical distribution of the parasite than previously considered. However, *D. nihonkaiense* has not been confirmed from other than three species of Pacific salmons (*O. masou masou*, *O. gorbuscha*, *O. keta*) in Japan and Far East Russia so far. There has been no

report of *D. nihonkaiense* larvae from any imported/exported Pacific salmons. To clarify the geographical distribution and the host range of *D. nihonkaiense*, more extensive fieldwork on Pacific salmons, especially along the Pacific coast of North America, is needed.

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Figure legend

Fig. 1. The strobila and the egg obtained in the present case. (A) Whole-mounted specimen of the strobila with multiple reproductive organs.

(B) Transverse section of the strobila stained with hematoxylin and eosin. Arrowheads show the margins. c: cirrus. cs: cirrus sac. u: uterus. Bar = 1 mm. (C) Schematic diagram of the strobila. Solid lines and broken lines show anterior and posterior margins of proglottidsr respectively. (D) The egg with an apical knob. Bar = 20 μm.