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First confirmed human case of *Diphyllobothrium stemmacephalum* infection and molecular verification of the synonymy of *Diphyllobothrium yonagoense* with *D. stemmacephalum* (Cestoda: Diphyllobothriidea)☆

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ABSTRACT

Several tapeworm species in the genus *Diphyllobothrium* Cobbold, 1858 have uncertain taxonomic positions, leading to taxonomic confusion as well as misdiagnosis of infections. Taxonomic revision based on DNA sequence analysis is considered necessary to resolve the taxonomy of several cases, including that between *Diphyllobothrium stemmacephalum*, the type species of the genus, and *Diphyllobothrium yonagoense*. *Diphyllobothrium yonagoense* was synonymized with *D. stemmacephalum* based on morphological observations by Andersen (1987), however no molecular studies have been undertaken to verify the validity of this synonymization.

In the present study, the first human case confirmed molecularly as *D. stemmacephalum* infection is reported, and the validity of the synonymization of *D. yonagoense* with *D. stemmacephalum* was assessed based on molecular phylogenetics.

Diphyllobothrium stemmacephalum and *D. yonagoense* grouped into the same clades with high bootstrap confidence values for both *cox1* and *nad3*. Genetic distances between the two taxa were very small (0.000–0.012 and 0.000–0.017 for *cox1* and *nad3*, respectively) and were considered to fall within the range of intraspecific variation. Using these molecular analyses, this study verified molecularly that *D. yonagoense* is a junior synonym of *D. stemmacephalum*. Further, the closer phylogenetic relationship between *D. stemmacephalum* and *Diplogonoporus* species rather than other diphyllobothriids, including *Diphyllobothrium nihonkaiense* and *Diphyllobothrium latum*, was corroborated. The genus name for *D. nihonkaiense* and *D. latum* is also discussed.

1. Introduction

The genus *Diphyllobothrium* Cobbold, 1858, contains 38 species [1,2], 11 to 14 of which, including *Diphyllobothrium latum* (Linnaeus, 1758) Lühe, 1910, *Diphyllobothrium nihonkaiense* Yamane et al., 1986, *Diphyllobothrium yonagoense* Yamane et al., 1981, *Diphyllobothrium dendriticum* (Nitzsch, 1824) Lühe, 1910 and *Diphyllobothrium pacificum* (Nybelin, 1931) Margolis, 1956 (*Adenocephalus pacificus* is currently accepted [4,5]), can cause diphyllobothriosis in humans [1,3–8]. On the other hand, the taxonomic relationships of several *Diphyllobothrium* species remain uncertain and are in need of clarification using molecular analyses [3,8,9]. One such taxonomic relationship in particular need of clarification is that between *Diphyllobothrium stemmacephalum* Cobbold, 1858, the type species of the genus, and *D. yonagoense*.

Diphyllobothrium stemmacephalum was first described based on broad tapeworms that were collected from a harbour porpoise (*Phocoena phocoena*) (Odontoceti: Phocoenidae) caught on the east coast of Scotland [10]. Since then, *D. stemmacephalum* has been confirmed in different Odontoceti species in other regions other than the type locality, including Europe [11–13], Russia [14], North America

Abbreviations: *cox1*, cytochrome *c* oxidase subunit 1 gene; *nad3*, NADH dehydrogenase subunit 3 gene; 18S rDNA, 18S ribosomal RNA gene; PCR, polymerase chain reaction.

☆ Nucleotide sequences of *cox1*, *nad3* and 18S rDNA of *Diphyllobothrium stemmacephalum* from a human case (case 24) have been deposited in the DNA Data Bank of Japan under accession numbers LC042231, LC042538 and LC042537, respectively. Nucleotide sequences of *cox1* and *nad3* of three *Diphyllobothrium yonagoense* isolates (registered as *D. stemmacephalum*) have been deposited under accession numbers LC090627–LC090629 and LC071716–LC071718, respectively (see Tables 3 and 4).

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[15–17] and Japan [18,19]. However, no confirmed human cases of *D. stemmacephalum* infection have been reported to date.

Diphyllobothrium yonagoense was described based on a diphyllobothriid tapeworm collected from a Japanese patient in Yonago City, Tottori Prefecture [20], and 13 subsequent human cases have been reported in Japan [21–28] and Korea [29] (Table 1). *Diphyllobothrium yonagoense* was collected from a Risso's dolphin (*Grampus griseus*) (Odontoceti: Delphinidae) from the Sea of Japan off Shimane Prefecture, Japan [30]. The taxonomic relationship between *D. stemmacephalum* and *D. yonagoense* was previously unclear [31,32]. *Diphyllobothrium yonagoense* was subsequently synonymized with *D. stemmacephalum* based on morphological observations [33], and the synonymization is currently accepted [3]. However, no studies have been undertaken to clarify the molecular-phylogenetic relationship between these taxa and the validity of the synonymization.

We recently encountered a human case (case 24, Table 1) of *D. stemmacephalum* infection in Japan, which we briefly reported in the Japanese literature [34]. In the present study, therefore, we present a detailed account of the human case (case 24) and clarify the validity of the synonymy of *D. yonagoense* with *D. stemmacephalum* based on molecular-phylogenetic analyses using mitochondrial DNA markers (*cox1* and *nad3*).

The analysis using parasite material from a patient (case 24) was performed according to a protocol (No. 589) approved by the Medical and Ethical Committee of National Institute of Infectious Diseases, Tokyo.

2. Materials and methods

2.1. Diphyllobothriid tapeworms examined in this study

In this study, diphyllobothriid proglottids obtained from four Japanese patients were examined. Three *D. yonagoense* specimens were obtained from 50-, 30- and 51-year-old Japanese men in cases 8, 10 and 23 [28], respectively (Table 1), all of whom resided in Kochi Prefecture, Japan. In case 8, the patient was admitted to a local hospital carrying naturally expelled proglottids in 1982 and the tapeworm was identified as *D. yonagoense* based on the presence of operculated eggs with a thick eggshell, dense and deep pits on the egg surface, numerous prominent and longitudinal furrows on the proglottid surface, and the shape of the uterus, all of which differed from those of *D. latum*.

The tapeworm passed by case 10 was 9.8 m in length and 1.8 cm in width and was also identified morphologically as *D. yonagoense*. The tapeworm and eggs obtained from case 23 were well documented [28]. The tapeworm from case 24 was identified as *D. stemmacephalum* by DNA-based analysis [34] (Table 1). The *D. yonagoense* specimens were preserved in 10% formalin for years, whereas the *D. stemmacephalum* specimen was fixed in 70% ethanol.

2.2. Morphological observations under light and scanning electron microscopy

Morphological observations of strobila from case 24, including of eggs in the uterus, were carried out by light and scanning electron microscopy (SEM). A part of the strobila was processed as paraffin-embedded specimens, and thin sections were stained with Masson's trichrome and/or hematoxylin eosin (HE). To observe the uterus, the proglottids were gently layered between two slide glasses and dehydrated by passing through an ethanol series and then into xylene before staining with boric acid-carmine. The proglottids and eggs for SEM were re-fixed in 2.5% PBS-buffered glutaraldehyde and processed by dehydration through an ethanol series. The specimens were subjected to critical-point drying in a liquid CO_2 dryer (HCP-2, Hitachi, Japan), coated with 1% osmium tetroxide with a model Neoc-ST osmium plasma coater (Meiwaforsis, Tokyo, Japan) and then observed using SEM (Model SU6600, Hitachi, Japan).

2.3. DNA preparation, PCR amplification, DNA sequencing and molecular-phylogenetic analyses

Genomic DNA was extracted from *D. stemmacephalum* proglottids in case 24 using DNeasy Blood & Tissue kits (Qiagen, Germany) according to the manufacturer's instructions. For the formalin-fixed *D. yonagoense* samples, small pieces of proglottids were washed several times with sterilized distilled water, cut into smaller pieces and lysed overnight at 56 °C with occasional homogenization using a small mortar and pestle before being used for DNA extraction using the above kit.

For the *D. stemmacephalum* samples, full-length *cox1*, *nad3* and 18S rDNA were amplified by PCR using the primers shown in Table 2 and *TaKaRa Ex Taq*® Hot Start Version (Takara Bio, Shiga, Japan) as described previously [35,36]. For the *D. yonagoense* samples, the short fragments of *cox1* and *nad3* were amplified by PCR using the primer pairs indicated with asterisks in Table 2 and KOD FX DNA polymerase (Toyobo, Suruga, Japan). The amplification conditions consisted of an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of 98 °C for 10 s, 56 °C for 30 s, 72 °C for 1 min, and a final extension step of 72 °C for 5 min. Amplicons were cleaned using ExoSAP-IT® (Affymetrix/USB, CA) and used as templates for direct DNA sequencing. Samples for DNA sequencing were prepared using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Life Technologies, Foster City, CA) and run on a 3730 x l DNA Analyzer (Life Technologies).

Genetic distance (d) values were calculated by Kimura's 2-parameter model, which is considered to be suitable for estimating genetic distances of mitochondrial DNA [37]. Phylogenetic trees for *cox1* and *nad3* were constructed using the maximum likelihood algorithm with Hasegawa-Kishino-Yano (HKY) + G and HKY + I models, respectively, with the lowest Bayesian information criterion scores (MEGA 6, www.megasoftware.net). Clades were assessed by bootstrap resampling with 1000 replicates.

3. Results

3.1. A human case due to D. stemmacephalum (case 24)

The patient was a 39-year-old Japanese man living in Kanagawa Prefecture, Japan. He first passed a flat, whitish, string-like worm while defecating on January 4, 2015. Two days later, he passed another strobila measuring 2.4 m long with a maximum width of 1.6 cm (Fig. 1). The patient was first referred to Red Cross Hadano Hospital and then admitted to Musashino Red Cross Hospital where he was diagnosed with diphyllobothriosis. On admission, the patient was asymptomatic except for passing the strobila. Clinical laboratory data were normal: red blood cells, $506 \times 10^4/\mu l$; white blood cells, $65 \times 10^2/\mu l$; eosinophil count, 2.8%; hematocrit, 44.5%; hemoglobin, 15 g/dl; and vitamin B_{12}, 359 pg/ml. Vitamin B_{12}-deficient anemia was not observed. On January 27, 2015, the patient was administered a single dose of 600 mg of praziquantel and was treated successfully. No further proglottids have been passed as of May 2016.

3.2. Morphological description of the D. stemmacephalum

The naturally passed strobila was slightly fleshy in appearance and the scolex had been lost (arrow, Fig. 1). Although the posterior proglottids were partially fenestrated (arrowhead, Fig. 1), no gravid segments with 2 or 3 sets of reproductive organs in a single segment were observed. Numerous prominent and longitudinal furrows were observed on the ventral and dorsal surfaces of the progloottids (Fig. 2-A). In the medial sagittal sections of the gravid proglottids, a pyriform cirrus sac (417 × 347 μm) was connected posterodorsally to thick-walled,