■ **CASE REPORT**

Four Human Cases of *Diphyllobothrium nihonkaiense* (Eucestoda: Diphyllobothriidae) in China with a Brief Review of Chinese Cases

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Abstract: We described 4 human infection cases of zoonotic fish-tapeworm, *Diphyllobothrium nihonkaiense*, identified with morphological and molecular characters and briefly reviewed Chinese cases in consideration of it as an emerging parasitic disease in China. The scolex and mature and gravid proglottids of some cases were seen, a rosette-shaped uterus was observed in the middle of the mature and gravid proglottids, and the diphyllobothriid eggs were yellowish-brown in color and displayed a small knob or abopercular protuberance on the opposite end of a lid-like opening. The average size of the eggs was recorded as 62-67 × 42-45 μm. The parasitic materials gathered from 4 human cases were morphologically identified as belonging to the genera *Diphyllobothrium* and *Adenocephalus*. The phylogenetic analysis based on the nucleotide sequences of cytochrome *c* oxidase subunit 1 gene of the etiologic agents confirmed that the 4 cases were *D. nihonkaiense* infection. The finding of 4 additional *D. nihonkaiense* cases suggests that *D. nihonkaiense* might be a major causative species of human diphyllobothriasis in China. A combined morphological and molecular analysis is the main method to confirm *D. nihonkaiense* infection.

Key words: *Diphyllobothrium nihonkaiense*, case report, diphyllobothriasis, phylogenetic analysis, China

INTRODUCTION

Diphyllobothriasis is a fish-borne parasitic zoonosis caused by infection of the adult tapeworm belonging to the genus *Diphyllobothrium* [1]. It has been reported that 14 species are etiologic agents that can cause diphyllobothriasis, including *Diphyllobothrium nihonkaiense*, *D. latum*, and *Adenocephalus pacificus* (syn. *D. pacificum*) [1-3]. Diphyllobothriasis caused by *D. nihonkaiense* has been mostly reported in the eastern part of Asia, in particular Japan [4,5], South Korea [6], and the Far Eastern Federal District of Russia [7]. In Japan, Yamane et al. [8] established *D. nihonkaiense* as a new species in 1986. The infection has been perpetuated among urban people who eat sushi and sashimi. The incidence was particularly high in 2008 [5].

In Korea, *D. nihonkaiense* was first diagnosed in 2009, and there were about 62 cases previously diagnosed as *D. latum* which were confirmed to be actually *D. nihonkaiense* based on molecular analysis [6]. As the main infection sources of this tapeworm, salmonid fish, i.e., *Oncorhynchus masou*, *O. gorbuscha*, *O. keta*, and *O. nerka*, which inhabited in the northern Pacific Ocean, were reported in these countries [9,10].

Recently, this tapeworm infections have also been reported in Europe [11-13], North America [14,15], and New Zealand [16]. In mainland China, human *D. latum* infection was first found in Heilongjiang province in 1927 [17], and 22 human cases of diphyllobothriasis have been reported between 1927 and 2014 [18-22]. There were 14 *D. latum* cases in total until now, mainly distributed in Heilongjiang province, Beijing, Guangdong province, Tianjin, Fujian province, and Shanghai [18-21]. Human *D. nihonkaiense* infection was first found in Shanghai in 2008, when it was first morphologically misdiagnosed as *D. latum* infection; however, Chen et al. [22] discovered it as a *D. nihonkaiense* human case diagnosed definitively by molecular analysis of expelled proglottids. Total 8 human *D. nihonkaiense* cases were reported from 2008 to 2014; 1 case

Table 1. Summary on the human infection cases with *D. nihonkaiense* in China

Case no	Residence	Year of diagnosis	Place of eating fish	Suspected source of infection	Morphologic and molecular identificaton	Abroad or local infection	Reference
1	Shanghai/Japan	2008	Shanghai	raw salmon	*D. nihonkaiense*	local	22
2	Heilongjiang	2009	Heilongjiang	raw fish	*D. nihonkaiense*	local	22
3	Shanghai	2011	Shanghai	raw salmon	*D. nihonkaiense*	local	22
4	Shanghai	2011	Shanghai	raw salmon	*D. nihonkaiense*	local	22
5	Shanghai	2011	Shanghai	raw salmon	*D. nihonkaiense*	local	22
6	Heilongjiang	2012 to 2014	Harbin	raw or undercooked fish	*D. nihonkaiense*	local	21
7	Heilongjiang	2012 to 2014	Fuyuan	raw or undercooked salmon	*D. nihonkaiense*	not sure	21
8	Heilongjiang	2012 to 2014	Harbin	raw fish dishes	*D. nihonkaiense*	local	21

in 2008 [22], 1 case in 2009 [22], 3 cases in 2011 [22], and 3 cases between 2012 and 2014 [21] (Table 1).

The main reason for *D. nihonkaiense* infection is that the salmon has become an increasingly important commodity globally, and the salmon trade has increased markedly due to rapid advances in international and domestic transport systems. This results in an increase in the occurrence of diphyllobothriasis as an emerging parasitic disease in regions where it previously did not exist. The infection occurs through the consumption of raw or undercooked fish containing the larval forms called plerocercoids. Some species can grow as long as 2-15 m in the human small intestine. Typical features of *D. nihonkaiense*, as the same as *D. latum*, is the spoon-like scolex with a deep groove and the mature and gravid proglottids having a rosette-shaped uterus [17]. Complete mitochondrial genomes of *D. nihonkaiense* and *D. latum* have been demonstrated recently, these genomes can distinguish the 2 species and rendered species diagnosis more reliable [5]. Clinical manifestations of diphyllobothriasis are generally mild and not serious. Abdominal discomfort, abdominal pain, and defecating "inch-sized white worms" have been reported [23].

The aim of this study is to report 4 human infection cases of zoonotic fish-tapeworm, *D. nihonkaiense*, identified with morphological and molecular characters and briefly reviewed Chinese cases with a consideration as an emerging parasitic disease in China.

CASE RECORD

The first case was a 43-year-old woman residing in Heilongjiang province, who expelled several fragments of a flat white worm in her feces. The patient stated that she frequently consumed raw or undercooked fish, such as the salmon, especially when she worked in Russia in 2012. She also consumed a lot of raw salmons in July and August in 2013 and 2014, when the salmons migrate from the ocean and swim to the upper reaches of rivers where they spawn. The patient caught the salmon in the river and ate the fish raw. She also had a history of eating other raw freshwater fish. Based on the morphology of the strobila and eggs, the sample was diagnosed as belonging either to *Diphyllobothrium* or *Adenocephalus*. On 29 March 2015, the patient orally took Chinese traditional medicine (decoction of betel nut and pumpkin seed) and $MgSO_4$ solution for catharsis. After 4.5 hr, a cestode of about 3 m in length was successfully excreted, though the scolex was missing.

The second case was a 40-year-old man living in Shanghai, who liked eating raw or undercooked salmon and had never traveled abroad. In March 2015, he consumed nearly 1 kg of raw salmon in a Japanese restaurant in Shanghai and passed some alive white proglottids in his feces 2 months later. After ingesting a decoction of betel nut and pumpkin seed, he expelled a 3.2 m long tapeworm, with the scolex morphologically identified as belonging to the genus *Diphyllobothrium*.

The third case was a 42-year-old Chinese man who spent most of his life living in Canada, where he ate a lot of raw or undercooked salmon and other sea fish. The patient expelled some fragments of a flat white worm in his feces in January 2016 when he came back to Shanghai to visit relatives. Based on the morphology of the strobila and eggs, the sample was diagnosed as belonging either to *Diphyllobothrium* or *Adenocephalus*. After he was orally administered with praziquantel, segments of proglottids were expelled from his anus. All proglottids were sent for DNA-PCR analysis.

The fourth case was a previously healthy 36-year-old man residing in Shanghai, who expelled some fragments of a flat white worm in his feces since October 2015. The patient stated that he likes to eat raw or undercooked fish, such as the salmon and trout. Based on the morphology of the strobila and

eggs, infection with a diphyllobothriid was strongly suspected. A single dose (20 mg/kg) of praziquantel and 250 ml of citrate magnesium were administered orally. The strobila was preserved in 70% ethanol and sent to our institute for further analysis.

The study was authorized by the ethical committee of Institute of Parasitic Diseases (IPD), Chinese Center for Disease Control and Prevention (China CDC). All participants were informed about the study aims and procedures, following which they were given a written informed consent to participate. Collection of specimens (tapeworm) was conducted with approval from the Ethics Committee of IPD, China CDC.

The parasitic material gathered was repeatedly washed in physiological saline solution, macroscopically examined and measured, explored by microscopy, and morphologically identified based on morphological characters [17]. Several proglottids of each tapeworm were preserved in 70% ethanol at 4°C until DNA extraction.

Genomic DNA was extracted from the proglottids excreted by each case using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) was amplified by PCR, according to the method previously described by Yamasaki and Kuramochi [16]. New primer pairs were constructed as follows: Diphyllo trnW/F (5′-ACAGTGGGTTTAGATGTAAAGACGGC-3′) and Diphyllo trnT/R (5′-AGTATTCTAATTAAACTATAAAGCC-3′).

All PCR products positive for *Diphyllobothrium* were sequenced with PCR primers at each locus after being purified using an ABI PRISM® 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA) and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The nucleotide sequences obtained in the present study were subjected to a BLAST search (http://www.ncbi.nlm.nih.gov/blast), and were analyzed and aligned with each other. *Diphyllobothrium* reference sequences were downloaded from GenBank using the program Clustalx 1.83 (http://www.clustal.org). A neighbor-joining tree was constructed at cox1 loci using the program MEGA 4 (http://www.megasoftware.net) based on the evolutionary distances calculated using Kimura's 2-parameter method. The reliability of the tree was assessed by bootstrapping with 500 replicates.

All worms recovered from cases were grossly revealed typical long tapeworm proglottids, and their each segment was wider than long. The tapeworms from the 1st and 2nd cases were 3.0 m and 3.2 m in length, respectively. The scolex of the tape-

worm from case 1 was missing, only mature and gravid proglottids were seen. The tapeworm of case 2 was integral, and the scolex and mature and gravid proglottids were seen. From cases 3 and 4, only segments of proglottids were obtained. The tapeworm from case 2 was used for carmine staining and further observation. Using an anatomical lens, it was determined that the scolex from case 2 was spoon-like with a deep groove (Fig. 1A, B). A rosette-shaped uterus was observed in the middle of the mature and gravid proglottids. *Diphyllobothrium* species eggs were also detected (Fig. 1C). The diphyllobothriid eggs were yellowish-brown in color and displayed a small knob or abopercular protuberance on the opposite end of a lid-like opening. Twenty eggs from each sample were measured using a microscope. The average size of the eggs was recorded as 65×44 μm (case 1), 63×42 μm (case 2), 67×45 μm (case 3), and 62×43 μm (case 4). The eggs from case 4 are shown as an example in Fig. 1D.

PCR products of cases 1 to 4 were all positive, and all PCR products positive for *Diphyllobothrium* were sequenced with PCR primers (Diphyllo trnW/F and Diphyllo trnT/R). The lengths of the partial *cox1* sequences (*pcox1*) of the samples were 1,566 bp. Molecular diagnostics confirmed that all parasitic material belonged to the species *D. nihonkaiense*. The sequences for cases 1 and 2 are available in the DNA Data Bank of Japan, with accession nos. LC070677 and LC070678, respectively. Meanwhile, sequences for cases 3 and 4 are available in GenBank, with accession nos. KU984425 and KU984426, respectively.

Topologies of *pcox1* sequences inferred by neighbor-joining methods (NJ methods) with different building strategies and/or different distance models were found to be similar (Fig. 2). The phylogenetic tree consisted of 2 large clades; the first one contained the genus *Diphyllobothrium* and the second contained *Spirometra*. In the first clade, the 4 patient's samples were included in the *D. nihonkaiense* family with a high bootstrap value (>50%). In the second clade, *Spirometra* samples were clustered together. This clustering is in agreement with the results of traditional classifications. *Taenia solium* was used as an outgroup (Table 2).

DISCUSSION

D. latum and *D. nihonkaiense* are almost indistinguishable morphologically. As recently reported [22], the 5 human cases of diphyllobothriasis in China were first diagnosed morpho-
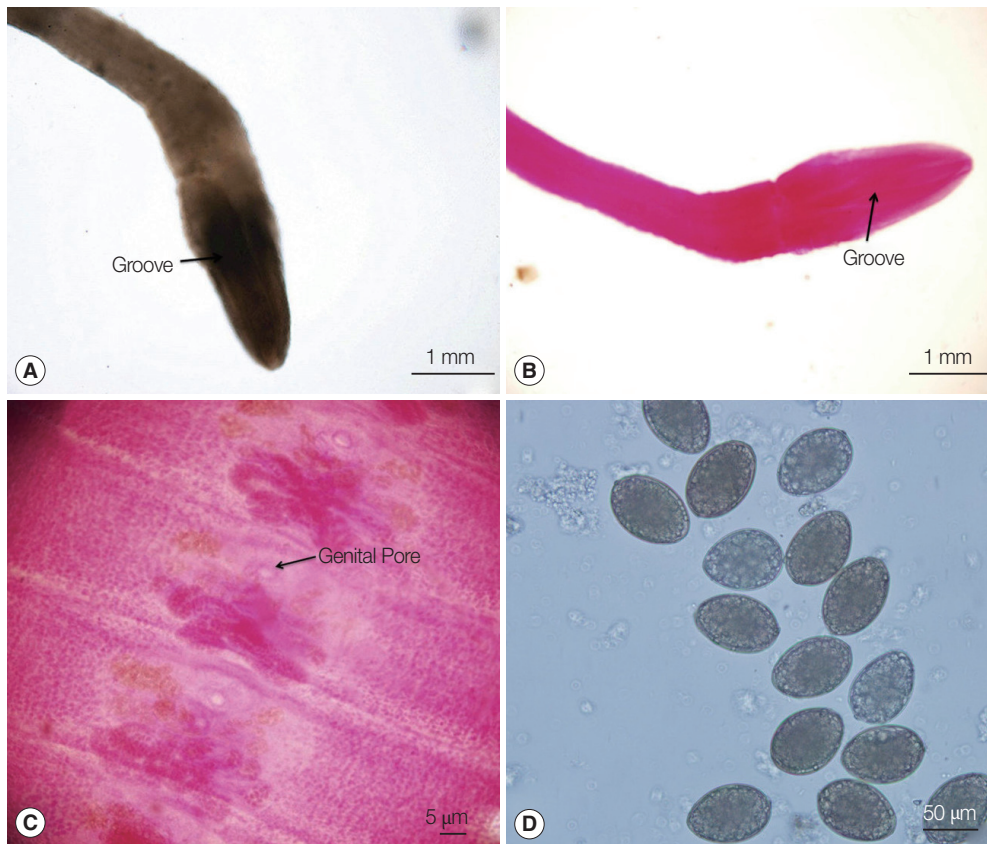
Fig. 1. Morphological features of worms under microscopic examinations. (A) The scolex from case 2. (B) Carmine-stained scolex from case 2. (C) Gravid proglottids stained with acetocarmine from case 2. (D) Eggs detected in fecal samples of case 4.
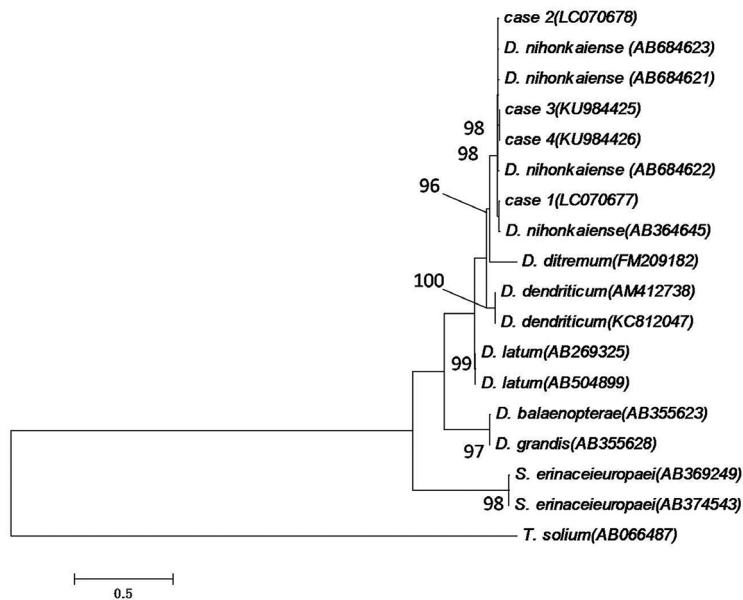

Fig. 2. A phylogenetic tree constructed using the maximum likelihood algorithm with HKY+G model on the basis of the complete *cox1* sequences of the isolates from the 4 Chinese cases and related *Diphyllobothrium* species. The numbers of the nodes are bootstrap values (500 replicates). *Taenia solium* was used as an outgroup. The scale bar indicates the number of base substitutions per site.

Table 2. Samples of *Diphyllobothrium* spp., *Diplogonoporus* spp., *Spirometra* spp., and *Taenia* spp. used in the present study, as well as their GenBank accession numbers for sequences of partial mitochondrial cytochrome c oxidase subunit 1 gene (*pcox1*)

Sample codes	Geographic origin	Host	Species	GenBank accession number (*pcox1*)
Case 1	Heilongjiang, China	*Homo sapiens*	*Diphyllobothrium nihonkaiense*	LC070677
Case 2	Shanghai, China	*Homo sapiens*	*D. nihonkaiense*	LC070678
Case 3	Canada	*Homo sapiens*	*D. nihonkaiense*	KU984425
Case 4	Shanghai, China	*Homo sapiens*	*D. nihonkaiense*	KU984426
CHN-005	Shanghai, China	*Homo sapiens*	*D. nihonkaiense*	AB684623
CHN-002	Japan	*Homo sapiens*	*D. nihonkaiense*	AB684621
CHN-003	Shanghai, China	*Homo sapiens*	*D. nihonkaiense*	AB684622
	Asahikawa, Japan	-	*D. nihonkaiense*	AB364645
TS02/32	Scotland, United Kingdom	*Salvelinus alpinus*	*Diphyllobothrium ditremum*	FM209182
	-	*Homo sapiens*	*Diphyllobothrium dendriticum*	AM412738
CZ49	Czech Republic	*Homo sapiens*	*D. dendriticum*	KC812047
	-	*Homo sapiens*	*Diphyllobothrium latum*	AB269325
	Santiago, Chile	*Homo sapiens*	*D. latum*	AB504899
D3	Sanriku-Oki, Japan	-	*Diphyllobothrium balaenopterae*	AB355623
DJ1	Tokyo, Japan	-	*Diplogonoporus grandis*	AB355628
	Tokyo, Japan	*Elaphe quadrivirgata*	*Spirometra erinaceieuropaei*	AB369249
	Japan	*Elape quadrivirgata*	*S. erinaceieuropaei*	AB374543
	Thailand	-	*Taenia solium*	AB066487

Table 3. Summary of the human infection cases with *D. nihonkaiense* in China of this study

Case no	Residence	Year of diagnosis	Place of eating fish	Suspected source of infection	Morphologic and molecular identification	Abroad or local infection	Reference
Case 1	Heilongjiang	2015	Russia	raw salmon	*D. nihonkaiense*	abroad	This study
Case 2	Shanghai	2015	Shanghai	raw salmon	*D. nihonkaiense*	local	This study
Case 3	Shanghai/Canada	2016	Canada	raw salmon	*D. nihonkaiense*	abroad	This study
Case 4	Shanghai	2016	Shanghai	raw salmon	*D. nihonkaiense*	local	This study

logically as *D. latum* infection. However, all tapeworms were confirmed to be *D. nihonkaiense* by molecular analysis [22]. The 4 additional *D. nihonkaiense* cases in this study were also confirmed to be *D. nihonkaiense* by analysis of cox1 gene. Given the morphological similarities among diphyllobothriid species, molecular analysis is an indispensable method for accurately identifying the correct species, and helpful not only for avoiding diagnostic confusion but also for facilitating the acquisition of reliable epidemiologic and epizootic information.

Adding 4 new cases in this study, total 12 human cases were reported in China in a short span of 8 years from 2008 to 2016; 10 of the 12 *D. nihonkaiense* cases were local infection, and 2 of them were abroad infection (Tables 1, 3). For case 1 of this study, the patient stated that she frequently consumed raw or undercooked fish when she worked in Russia from 2012 to 2014. For case 3, the patient ate a lot of raw or undercooked salmon and other sea fish in Canada. The number of

D. nihonkaiense cases is increasing year by year, especially in Shanghai. However, China is not a location where intermediate hosts are commonly distributed, and most of the salmon is imported. United States, Chile, Russia, Norway, and Japan were the top 5 countries that exported salmons to China in 2014 [24]. The 4 recent cases of *D. nihonkaiense* infection reported in Shanghai likely reflect the changes in the raw salmon-eating habits of people and preferences for specialties, such as sushi and sashimi, which are staples of healthy Japanese diets [15,16,22].

In this study, 2 of the patients (cases 1,2) orally took Chinese traditional medicine (decoction of betel nut and pumpkin seed) and $MgSO_4$ solution for catharsis. Two others (cases 3,4) orally administered a single dose of praziquantel (10 mg/kg). It was similar to the dose used to treat *D. latum* infection [17,23]. A decoction of betel nut and pumpkin seed has been recommended for the treatment of *D. nihonkaiense* and other tapeworm infections in China with a long time using, good ef-

fect, and good compliance [17]. A single dose of praziquantel is also recommended, but for some of the patients, 2 doses are thought to be necessary [25].

A limitation of this study was the small number of cases evaluated. Moreover, the species of intermediate host of *D. nihonkaiense* which the patients had eaten could not be obtained and evaluated. Because it took a long time when the patients were diagnosed and it is difficult to know exactly where the salmon the patients had eaten were from. In conclusion, studies on Pacific salmons as a potential source of infection for *D. nihonkaiense* in China should be conducted. More people should be made aware about the harmful effects of eating raw or undercooked fish [26]. *D. nihonkaiense* infection may become a new emerging parasitic disease in China.

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CONFLICT OF INTEREST

The authors declare that they have no competing interest.

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