■ ORIGINAL ARTICLE

Molecular Identification of *Diphyllobothrium nihonkaiense* from 3 Human Cases in Heilongjiang Province with a Brief Literature Review in China

Weizhe Zhang[1], Fei Che[2], Song Tian[3], Jing Shu[1], Xiaoli Zhang[1,*]

[1]Department of Parasitology, Harbin Medical University, Harbin, Heilongjiang, China; [2]Department of Neurology, the Second Affiliated Hospital of Qiqihar Medical University, Qiqihar, Heilongjiang, China; [3]Department of Animal Health Supervision, Heilongjiang Provincial Bureau of Animal Husbandry and Veterinary, Harbin, Heilongjiang, China

Abstract: Human diphyllobothriasis is a widespread fish-borne zoonosis caused by the infection with broad tapeworms belonging to the genus *Diphyllobothrium*. In mainland China, so far 20 human cases of *Diphyllobothrium* infections have been reported, and the etiologic species were identified as *D. latum* and *D. nihonkaiense* based on morphological characteristics or molecular analysis. In the present study, proglottids of diphyllobothriid tapeworms from 3 human cases that occurred in Heilongjiang Province, China were identified as *D. nihonkaiense* by sequencing mitochondrial cytochrome *c* oxidase subunit I (*cox1*) and NADH dehydrogenase subunit 5 (*nad5*) genes. Two different *cox1* gene sequences were obtained. One sequence showed 100% homology with those from humans in Japan. The remaining *cox1* gene sequence and 2 different *nad5* gene sequences obtained were not described previously, and might reflect endemic genetic characterizations. *D. nihonkaiense* might also be a major causative species of human diphyllobothriasis in China. Meanwhile, the finding of the first pediatric case of *D. nihonkaiense* infection in China suggests that infants infected with *D. nihonkaiense* should not be ignored.

Key words: *Diphyllobothrium nihonkaiense*, molecular identification, human, *cox1* gene, *nad5* gene

INTRODUCTION

Human diphyllobothriasis is a widespread fish-borne zoonosis caused by the infection with broad tapeworms belonging to the genus *Diphyllobothrium*. It has been estimated that 20 million people are affected globally [1]. Humans as the most suitable definitive host become infected through eating raw or undercooked freshwater or marine fish harboring plerocercoid larvae, which develop into adult tapeworms in the small intestine after ingestion. In most of cases, patients may remain asymptomatic until expulsion of proglottids of the adult parasite in feces, while others can suffer from mild digestive troubles, such as abdominal pain, diarrhea, and abdominal discomfort. Occasionally, *Diphyllobothrium latum* infection can cause malignant anemia due to the consumption of vita-

min B12 by the tapeworm. Several outbreaks caused by *Diphyllobothrium* had a significant impact on economy and public health [2,3].

The application of molecular methods based on the sequencing of mitochondrial and nuclear genes resulted in a better understanding of the genus *Diphyllobothrium*. To date, about 18 species of *Diphyllobothrium* have been reported to infect humans, with *D. latum* and *Diphyllobothrium nihonkaiense* being the most common [4]. Human diphyllobothriasis has been reported in Europe [3,5-7], North America [8,9], South America [2,10,11], and Asia [12-15]. In mainland China, a total of 20 human cases have been documented since the first report in 1927 (Table 1) [14-21]. All of them were initially identified as *D. latum* infection based on morphological characteristics of the worm, with 3 out of them being confirmed molecularly [14,21]; however, 5 out of them have been re-verified as *D. nihonkaiense* infection on the basis of molecular analysis recently [15].

D. nihonkaiense is relatively common in humans in Japan, Korea, and Far Eastern Federal District of Russia [7,22-24]. In addition, there have been case reports of human *D. nihonkaiense*

infection in France, Switzerland, Canada, New Zealand, and China [6,9,15,25-27]. In the present study, to understand the situation of *Diphyllobothrium* infection in Heilongjiang Province, China, we identified the proglottids of diphyllobothriid tapeworms from 3 human cases in Heilongjiang Province, China by sequencing mitochondrial cytochrome *c* oxidase subunit I (*cox1*) and NADH dehydrogenase subunit 5 (*nad5*) genes.

MATERIALS AND METHODS

Ethics statement

This research study was approved by the Medical Ethics Review Committee of Harbin Medical University, Harbin, China. Meanwhile, the Medical Ethics Review Committee of Harbin Medical University exempted the individual informed consent targeting molecular identification of the proglottids of tapeworms because the specimens were obtained after they were excreted in the feces.

Cases and proglottids collection

Between 2012 and 2014, total 3 strobilae (a chain of proglottids) of diphyllobothriid tapeworms were collected from 3 human cases that occurred in Heilongjiang Province, the northeastern part of China. The first patient was a 27-year-old woman residing in Harbin, who expelled some fragments of a flat white worm in the feces. She stated that she frequently consumed raw or undercooked fish, such as salmons, and had never traveled abroad. The second patient was a 39-year-old woman inhabiting Fuyuan, a county city near the Heilong River, who liked eating raw or undercooked salmons and had ever been to Russia. She passed some white living proglottids in the feces. The third patient was a 17-month-old girl born in Harbin. Her parents mentioned that they regularly ate raw fish dishes, such as salmons, and the girl was fed the delicious dishes once in a while. Her parents found some white flat segments in the girl's feces. The whole family did not have histories of overseas travel. Macroscopic examination of the 3 proglottids showed that each segment was wider than long. Microscopic examination of the fecal samples of 3 patients showed the eggs that were yellowish in color and elliptical in shape, with a broad operculum at one end, and belonged to *Diphyllobothrium* spp. eggs. Several proglottids of each tapeworm were preserved in 70% ethanol at 4°C until the DNA extraction.

DNA extraction and PCR amplification

Prior to DNA extraction, each proglottid specimen was washed 3 times with PBS to remove ethanol. The genomic DNA of *Diphyllobothrium* was extracted from approximately 25 mg proglottid tissue of each tapeworm using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer-recommended procedures. The DNA was eluted in 200 μl of AE elution buffer and was stored at -20°C until further use in PCR analysis. The mitochondrial *cox1* and *nad5* genes of *Diphyllobothrium* were amplified using previously published primers, respectively [14]. The reaction mixtures contained 2 μl DNA, 0.1 mM premixed solution of dNTP, 5 *p*mol of each primer, 2.5 μl PCR buffer (10×), 0.5 U of TaqDNA polymerase, and enough water for the final volume of 25 μl. Approximately 885 bp of the *cox1* gene and 1,028 bp of *nad5* gene were amplified, respectively, with the same cycle parameters as follows: an initial denaturation step at 94°C for 3 min; 35 cycles at 94°C for 1 min, 50°C to 60°C for 30 sec, and 72°C for 1 min 30 sec; followed by a final extension step at 72°C for 10 min [28]. Each DNA preparation was analyzed at least 3 times by PCR at both *cox1* and *nad5* loci. All PCR amplifications were run with non-template water control. All amplicons were fractionated in 1.5% agarose gel electrophoresis and visualized under UV light after ethidium bromide staining.

Nucleotide sequencing and molecular analysis

All PCR products of 2 genes positive for *Diphyllobothrium* were sequenced with the PCR primers at each locus after being purified on an ABI PRISM™ 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA), using a Big Dye Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems). Sequence accuracy was confirmed by 2-directional sequencing. Nucleotide sequences obtained in the present study were subjected to BLAST searches (http://www.ncbi.nlm.nih. gov/blast/) and then aligned and analyzed with each other. *Diphyllobothrium* reference sequences were downloaded from GenBank using the program Clustal X 1.83 (http://www.clustal. org/). Two neighboring-joining trees were constructed at *cox1* and *nad5* loci, respectively, using the program Mega 5 (http://www.megasoftware.net/) on the basis of the evolutionary distances calculated by Kimura-2-parameter model. The reliability of the 2 trees was assessed using the bootstrap analysis with 1,000 replicates.

RESULTS

All 3 proglottids were identified as *D. nihonkaiense* based on *cox1* and *nad5* genes. The representative nucleotide sequences obtained in the present study were deposited in the GenBank database under the following accession nos.: KP273585 and KP273586 (*cox1*), and KP273587 and KP273588 (*nad5*).

Two different *cox1* gene sequences were obtained from 3 proglottids of tapeworms. One sequence (KP273585) (n = 2) showed 100% homology with those from humans in Japan (AB781787 and AB573405). The remaining one (KP273586) was novel and had 99.8% similarity with the reference sequence AB781787, with 2 base differences at nucleotide sites 540 (T to C) and 789 (T to C). However, both codons (CGC and CGT), and both codons (TGC and TGT) could be translated into the amino acids arginine (R) and cystine (C), respec-

tively (Table 1). Two *nad5* gene sequences obtained were not described previously and had 99.8% and 99.7% similarity with the complete genomic sequences of *Diphyllobothrium* (AB268585 and EF420138), respectively, with 1 base difference between one another. One sequence (KP273587) (n = 2) had 2 base differences at nucleotide sites 624 (C to T) and 816 (G to A), and the remaining one (KP273588) had 3 base differences at nucleotide sites 315 (A to G), 624 (C to T), and 816 (G to A) compared to the reference sequence AB268585; however, each both codons (TTA and TTG; TTC and TTT; TCG and TCA) could be translated into the amino acids leucine (L), phenylalanine (F) and serine (S), respectively (Table 1).

Phylogenetic analyses based on 2 neighbor-joining trees at *cox1* and *nad5* loci also showed that all 3 proglottids of tapeworms in the northeast of China (=Heilongjiang Province) were *D. nihonkaiense* (Fig. 1). By analysis of the *cox1* gene se-

Table 1. Differences in bases and amino acids at *cox1* and *nad5* loci of *D. nihonkaiense*

Gene	Accession no./case	Homology (%)	Codon (amino acid[a])/Nucleotide position[b]		
cox1	AB781787	Ref. sequence	CGT(R)/540	TGT(C)/789	
	KP273585/ case 1,2	100	CGT(R)/540	TGT(C)/789	
	KP273586/ case 3	99.8	CGC(R)/540	TGC(C)/789	
nad5	AB268585	Ref. sequence	TTA(L)/315	TTC(F)/624	TCG(S)/816
	KP273587/ case 1,2	99.8	TTA(L)/315	TTT(F)/624	TCA(S)/816
	KP273588/ case 3	99.7	TTG(L)/315	TTT(F)/624	TCA(S)/816

[a]R, C, L, F, and S represent arginine, cystine, leucine, phenylalanine, and serine, respectively.
[b]Nucleotide position numbers according to AB781787 and AB268585, with the beginnings of the coding region of *cox1* and *nad5* loci as position no.1 respectively.
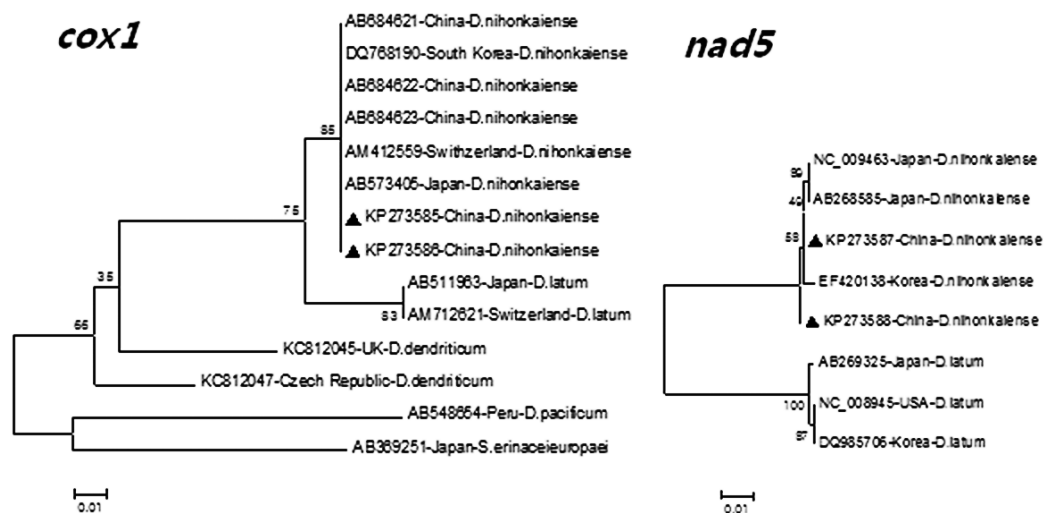

Fig. 1. Comparison of phylogenies of different species within *Diphyllobothrium* based on *cox1* and *nad5* gene sequences. Two phylogenetic trees were constructed using the neighbor-joining distance method analysis with a Kimura-2-parameter model. The reliability of 2 trees was assessed using the bootstrap analysis with 1,000 replicates. The triangles indicate the sequences of *Diphyllobothrium* from the present study.

Table 2. Variations at polymorphic sites within *cox1* gene sequences of *D. nihonkaiense* in China

GenBank accession no.[a]	Nucleotide at position									
	87	96	373[b]	480	504	540	600	642	717	789
KP273585	G	C	A	A	T	T	T	A	C	T
KP273586	G	C	A	A	T	C	T	A	C	C
AB684621	G	T	G	A	T	T	C	C	C	T
AB684622	G	C	A	G	T	T	T	A	T	T
AB684623	A	C	A	A	C	T	T	A	C	T

[a]The shorter *cox1* gene sequences of *D. nihonkaiense* (AB684624 and AB684625) were not included due to DNA degradation caused by the fixative.
[b]The corresponding codons (ATC and GTC) at the variant site can be translated into different amino acids isoleucine and valine, respectively.

Table 3. Cases of human diphyllobothriasis in mainland China from 1927 to 2014

Place of residence	No. of cases	Suspected source of infection (no.)	Morphological identification (no.)/Molecular identification (no.)	Ref.
Beijing	2	Unknown (2)	*D. latum* (2)/Untested (2)	[16,17]
Fujian	2	*Plecoglossus altivelis* (1) Raw salmon (1)	*D. latum* (2)/Untested (2)	[18,19]
Guangzhou	1	Unknown (1)	*D. latum* (1)/Untested (1)	[17]
Heilongjiang	7[a]	Raw fish (2) Raw salmon (1) Unknown (4)	*D. latum* (7)/*D. latum* (1), *D. nihonkaiense* (1), Untested (5)	[14,15,17,20]
Jilin	2	Raw fish (1) Raw salmon (1)	*D. latum* (2)/*D. latum* (1), Untested (1)	[14,17]
Shanghai	6	Raw salmon (5) Unknown (1)	*D. latum* (6)/*D. latum* (1), *D. nihonkaiense* (4), Untested (1)	[15,17,21]

[a]These cases did not include the 3 cases in this study.

quence (KP273586) with the previous sequence of *D. nihonkaiense* (AB684621-AB684623) in Shanghai, a total of 10 polymorphic sites were shown; however, different codons at different variant sites could be translated into the same amino acids, respectively, except at nucleotide sites 373, which codon (ATC) and codon (GTC) could be translated into amino acids isoleucine and valine, respectively (Table 2).

DISCUSSION

So far 20 human cases of *Diphyllobothrium* infections have been reported in mainland China since 1927 (Table 3) and the etiologic agents initially were identified as *D. latum* based on morphological characteristics, with 3 out of them were confirmed molecularly. Recently, 5 out of these cases have been reconfirmed as *D. nihonkaiense* infection by Chen et al. [15] for the first time on the basis of molecular analysis, this inconformity was considered that *D. latum* and *D. nihonkaiense* were almost indistinguishable morphologically, and laboratory workers usually identified the *Diphyllobothrium* species according to previously reported cases in the area.

In the present study, 3 tapeworm samples from human cas-

es of Heilongjiang Province were identified as *D. nihonkaiense* molecularly. The application of molecular markers based on species-specific suitable genes for accurately identifying *Diphyllobothrium* species is helpful in accumulating the geographical and epidemiological information associated to this parasite. As mitochondrial DNA is known to be more powerful than nuclear DNA due to its rapid sequence evolution [29], and the sequences of mitochondrial *cox1* and *nad5* genes of *Diphyllobothrium* spp. were available in the GenBank database, we applied *cox1* and *nad5* genes to identify the 3 tapeworms.

This is the second report of human infection with *D. nihonkaiense* in mainland China. In fact, for many years whenever eggs of *Diphyllobothrium* spp. in fecal smears or proglottids were observed in this area, the identification was made invariably as "*D. latum*". However, 3 *Diphyllobothrium* isolates in our study, in combination with the previous 5 isolates re-identified, a total of 8 *Diphyllobothrium* isolates have been identified as *D. nihonkaiense* molecularly in mainland China. Thus, it is strongly suggested that some previous diphyllobothriasis cases may have been misdiagnosed only based on morphological characteristics, and *D. nihonkaiense* may be the major causative species of human diphyllobothriasis in China. Similar results

have been reported in Korea. Most of the previous diphyllobothriasis cases were thought to be caused by *D. latum*; however, they were re-confirmed as *D. nihonkaiense* infections [22,30]. Thus, it is necessary to molecularly analyze for specific diagnoses of *D. nihonkaiense* and *D. latum* infection in humans.

D. nihonkaiense was first identified by Yamane et al. [31] in Japan in 1986. The cestode is also common in humans in Japan, Korea, and the Russian Far East [12,22-24]. Pacific salmons (*Oncorhynchus* spp.), especially the chum salmon (*Oncorhynchus keta*) and sockeye salmon (*Oncorhynchus nerka*) serve as its second intermediate hosts [7,32,33]. Plerocercoid larvae can be found in the musculature, liver, and gonads of these fish. A report showed that plerocercoid larvae of *D. nihonkaiense* with infection rates as high as 51.1% (24/47) were found in immature chum salmon commonly referred to as *tokishirazu* [34]. However, never plerocercoid larvae have been found in freshwater or marine fish in China. It is better to detect plerocercoid larvae in fish to find and identify the *Diphyllobothrium* species other than *D. latum* and *D. nihonkaiense* in China in the near future.

In the present study, since all 3 patients had the history of consumption of raw or undercooked salmons, it seems likely that the infection sources of *D. nihonkaiense* in the 3 patients might be salmons. However, it is difficult to identify the salmon species based on sashimi, sushi, and other salmon dishes. As the second patient had ever been to the Russian Far East, whether she became infected in China or Russia is unclear. It is surprising that the third patient was a 17-month-old girl. She is the first pediatric case of *D. nihonkaiense* infection in China, and is also the youngest patient infected with *D. nihonkaiense* based on molecular identification worldwide so far. It should not be ignored that infants can be infected with *D. nihonkaiense*.

In the present study, the *cox1* gene sequence (KP273586) was novel, with 2 base differences compared to the reference sequence AB781787; 2 *nad5* gene sequences were not described previously, having 99.8% and 99.7% similarity with the complete genomic sequences of *Diphyllobothrium* (AB268585 and EF420138), respectively (Table 1). However, these base substitutions were synonymous mutations and did not affect the respective amino acid translations due to the degeneracy of amino acids. The *cox1* gene sequence and 2 *nad5* gene sequences possibly reflected endemic genetic characterizations of *D. nihonkaiense* in China.

The cases of human diphyllobothriasis may increase with increasing consumption of salmons and other fish, and growing popularity of raw fish dishes, such as sashimi and sushi, in Heilongjiang Province, even in China. It seems that the general public is not aware of the potential risk for diphyllobothriasis posed by consumption of raw or uncooked freshwater or marine fish. Thus, it is necessary for the general public, especially food handlers and consumers to provide some information about the risk associated with eating habits to prevent more people from being infected with diphyllobothriasis. Freezing fish at -20℃ for at least 24 hr, or -35℃ for 15 hr, or cooking fish at 55℃ or more for at least 5 min is sufficient to kill plerocercoid larvae of *Diphyllobothrium* spp. possibly present in fresh fish to prevent transmission of the parasites.

ACKNOWLEDGMENT

CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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