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Human diphyllobothriosis in Taiwan: A review of cases and molecular evidence of *Dibothriocephalus nihonkaiensis*

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ABSTRACT

Diphyllobothriosis is an infectious disease caused by the consumption of raw freshwater or marine fish containing larvae of broad tapeworms (Diphyllobothriidae). In the present study, we critically reviewed all cases of human diphyllobothriosis reported from Taiwan, including unpublished reports from hospitals in Taipei. Genotyping based on mitochondrial DNA marker (*cox*1) confirmed that two of the recent cases were caused by *Dibothriocephalus nihonkaiensis*, which is not native to Taiwan and was probably imported with Pacific salmon infected with larvae of *D. nihonkaiensis*. The causative species previously reported in Taiwan could not be definitively confirmed. However, considering the distribution of *Dibothriocephalus latus*, which is not endemic in Taiwan, past cases diagnosed as *D. latus* are questionable.

1. Introduction

Tapeworms of the family Diphyllobothriidae Lühe, 1910 (Cestoda: Diphyllobothriidea) are known parasites of humans (*Adenocephalus*, *Dibothriocephalus*, *Diphyllobothrium* and *Spirometra*), with recent estimates of 15–20 million human cases worldwide (Kuchta et al., 2015; Waeschenbach et al., 2017). Of the dozens of recognized species of broad tapeworms, at least 15 species have been identified in humans (Scholz and Kuchta, 2016). Human diphyllobothriosis is a parasitic disease caused by consumption of raw fish infected with larvae (plerocercoids) of broad fish tapeworms (*Dibothriocephalus* and *Diphyllobothrium* spp.). Adult worms of *Dibothriocephalus latus* (syn. *Diphyllobothrium latum*) are the largest parasites of humans and can grow up to 17 m in length (Kuchta et al., 2023). Although most cases of diphyllobothriosis are asymptomatic, abdominal discomfort, diarrhea, vomiting, weakness, and weight loss may occur (Kuchta et al., 2023). The two most commonly reported species are *D. latus*, which was originally found mainly in temperate zones of the Holarctic and is transmitted by freshwater fishes such as perch (*Perca* spp.), pike (*Esox* spp.), pikeperch and walleye (*Sander* spp.), ruffe (*Gymnocephalus cernua*), and burbot (*Lota lota*), followed by *Dibothriocephalus nihonkaiensis* on the North

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Pacific coast (Dupouy-Camet and Peduzzi, 2004; Scholz and Kuchta, 2016; Kuchta et al., 2023). *Dibothriocephalus nihonkaiensis* was originally described from Japan and uses anadromous Pacific salmonids (*Oncorhynchus masou*, *O. keta*, *O. gorbuscha*, and *O. nerka*) as second intermediate hosts. It is the most common causative agent of diphyllobothriosis in the North Pacific coast of Asia (Russia, China, Japan and South Korea), North America (Canada and USA), but several imported cases have been reported in Europe, or New Zealand (Scholz and Kuchta, 2016).

Although the clinical signs of diphyllobothriosis caused by different species are similar, accurate species identification is important to determine the source of human infections. Morphological identification of clinical samples is usually impossible due to incomplete specimens and the morphological similarity of most taxa (Scholz and Kuchta, 2016). Therefore, reliable identification requires the use of molecular methods, for instance, complete sequence analysis of mitochondrial cytochrome *c* oxidase subunit I gene (*cox*1) (Waeschenbach et al., 2017).

In the present work, we provide a molecular identification of two recent cases based on partial cytochrome *c* oxidase subunit 1 (*cox*1) sequence analysis. We also critically reviewed all cases reported from Taiwan, including unpublished records from hospitals in Taipei.

2. Materials and methods

2.1. Materials

Two male patients, aged 38 (case 1) and 53 years (case 2), who complained of anal itching and excretion of noodle-like material (tapeworms) from the anus, visited Taipei Medical University Hospital (TMUH) in 2013 and 2016, respectively. The tapeworms were fixed in 10% buffered formalin or 96% ethanol, and stored at 5 °C. A scolex and the middle part of the strobila with proglottids were prepared for light and scanning electron microscopy (SEM) according to the methods described by Oros et al. (2010).

The studies involving human participants were reviewed and approved by Taipei Medical University, Joint Institutional Review Board – TMU-JIRB no. N202306060. Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

2.2. Molecular analysis

Genomic DNA was extracted from both formalin-fixed (case 1) and ethanol-fixed (case 2) samples using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The formalin-fixed sample was repeatedly washed in saline, lysed, and incubated at 70 °C for 30 min before genomic DNA extraction (Shi et al., 2002). The *cox*1 gene was amplified with the forward primer (D1F -5′-TGTGTGGGGGCATCATATGT-3′) and the reverse primer (D1R -5′-ATGATAAGGAACAGGAGCTCAGCATA-3′) (Park et al., 2015). The amplicons were 609 bp long, and mapped 878–1487 bp region of complete *cox*1 gene (1566 bp long) of the genus *Dibothriocephalus*. PCR was performed with initial denaturation at 95 °C for 5 min, 35 cycles at 94 °C for 1 min, annealing at 46 °C for 1 min, and extension at 72 °C for 1 min with final extension at 72 °C for 5 min. PCR amplicons were separated using a 2% agarose gel stained with SYBR Safe DNA stain in 0.5% TBE buffer, purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany), and sequenced by TRI-I Biotech Ltd. (Taipei, Taiwan). Sequences were checked for ambiguous positions in Geneious® (Kearse et al., 2012), identified using nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and deposited in the GenBank database (both NCBI, Bethesda, USA).

2.3. Phylogenetic analysis

The dataset for phylogenetic analysis included selected *cox*1 sequences from available congeneric taxa, i.e., *D. latus* (GenBank acc. nos. KY552871 and AB269325), *D. nihonkaiensis* (LC312466, AM412560, AB364645, AB268585, AB684623, KY000483 and AB924504), *Dibothriocephalus ditremus* (AB979518 and KY552872), *Dibothriocephalus ursi* (AB605763), and *Dibothriocephalus dendriticus* (KY552870 and AM412738); *Spirometra mansoni* (AB369251) represented the outgroup (see Waeschenbach et al., 2017). Alignment was performed with MAFFT ver.7.388 using the L-INS-I algorithm (Katoh and Standley, 2013). Optimal substitution models (1st codon: TN+F+I+G4, 2nd codon: TIM+F, 3rd: TN+F+I+G4) were estimated in ModelFinder using the AICc criterion (Kalyaanamoorthy et al., 2017). Phylogenetic relationships were expressed as a consensus tree constructed in IQ-Tree 2.0.5 using the maximum likelihood method with ultrafast bootstrap approximation (1000 replicates) (Minh et al., 2020).

3. Results

3.1. Human diphyllobothriosis in Taiwan (Table 1)

The first case was reported in 1956 by Frick et al. who found eggs of *Diphyllobothrium* in one of 1798 soldiers from Tainan (southern Taiwan) during a stool examination, followed by two more cases reported by Bergner in 1964 after eggs were found in stool samples from 143 residents of Taitung County. Another three cases were reported from Kaohsiung and Taipei between 2006 and 2017 (Chou et al., 2006; Lou et al., 2007; An et al., 2017), including one case identified as *D. latus* by multiplex PCR (An et al., 2017). From 2007 to 2011, five unpublished cases were detected at National Taiwan University Hospital (4 patients) and Tri-Service General Hospital (1 patient) in Taipei (Table 1). Three of them were identified from internal transcribed spacer sequences (ITS) in an unpublished MSc

thesis (Ro, 2011). Three more cases were recently discovered in a hospital in Taipei. Worms from two of them (2013 and 2016) show morphological features of *Dibothriocephalus* sp. (body with several hundred proglottids wider than an elongate scolex with a pair of bothria, i.e., dorsal and ventral longitudinal grooves, proglottids filled with numerous vitelline follicles in the cortex, numerous spherical testes in the medulla, a round cirrus-sac, the uterus tubular, forming several loops in the central part of proglottids, and genital pores on the ventral side of proglottids – Kuchta et al., 2015) when studied using a light microscope and a scanning electron microscope (SEM) (Fig. 1).

3.2. Molecular identification

The *cox*1 sequence analysis revealed two nearly identical sequences (pairwise divergence 0.2%, accession number OR625128 and OR625129). The phylogenetic tree showed the close genetic relationship with *D. nihonkaiensis* from USA (acc. no. KY000483) and Japan (acc. no. AB924504) (Fig. 2), which led us to identify our specimens as *D. nihonkaiensis.* Pairwise divergence (uncorrected *P* value) within the *D. nihonkaiensis* clade was 0–1.9%, whereas interspecific pairwise divergence between *D. nihonkaiensis* and certain congeners was much higher, i.e., 4.1–5.2% for *D. ursi*, 6.3–6.7% for *D. dendriticus*, 7.1–8.1% for *D. latus*, and 10.0–11.2% for *D. ditremus*.

4. Discussion

Diphyllobothriosis is an emerging infectious disease caused by consumption of raw or inadequately cooked fish containing larvae of *Diphyllobothrium/Dibothriocephalus* and also *Adenocephalus* species. In Asia, Japan appears to have the highest reported incidence (Arizono et al., 2009; Ikuno et al., 2018), particularly due to the high consumption of raw fish, including Pacific salmon, but the number of cases is also increasing worldwide (Scholz and Kuchta, 2016). Two cases of diphyllobothriosis caused by *D. nihonkaiensis* have been reported recently in Taiwan, and the likely source of infection is considered to be imported, chilled Pacific salmon due to the dietary habits of patients.

Most clinical cases reported from Taiwan have been identified as *D. latus* (see Table 1), but available data from other regions (Russia, Japan and South Korea) and the authors' experience cast doubt on this species identification because of the difficulty in distinguishing between *D. nihonkaiensis* and *D. latus* due to their morphological similarity and differences in geographic distribution (Dick et al., 2001). In addition, the second intermediate (fish) hosts of *D. latus* (mentioned earlier) are not native to Taiwan and are not imported for human consumption (Kuchta et al., 2019). An et al. (2017) identified a human isolate as *D. latus* using a multiplex PCR assay, but the reliability of this method should be verified because misidentification of *Adenocephalus pacificus (syn. Diphyllobothrium pacificum)* as *D. latus* has been confirmed (Kuchta et al., 2014). In addition, the ITS sequences used by Ro (2011) are not suitable for species identification of broad tapeworms (Waeschenbach et al., 2017).

The occurrence of exotic diphyllobothriosis in Taiwan cannot be verified because clinical samples are either unavailable or have been preserved in inappropriate fixatives for molecular identification. To avoid future confusion, clinicians are strongly advised to preserve specimens (proglottids or stool samples with eggs) in 90–96% molecular, i.e., non-denaturated ethanol, and store them for later genetic identification. Given the available information, including our new molecular data (Fig. 2) and the popularity of consuming imported Pacific salmon as in Taiwan, we hypothesise that some, if not all, cases of diphyllobothriosis previously reported in Taiwan were misdiagnosed and caused by *D. nihonkaiensis*, as confirmed in China, Japan, South Korea, eastern Russia and Singapore (Yamasaki et al., 2007; Cai et al., 2017; Ko et al., 2019; Kuchta et al., 2019). This assumption is supported by anamnestic data from some patients who admitted frequent consumption of imported Pacific salmon (Table 1).

Table 1
List of human diphyllobothriosis cases reported from Taiwan.

No.	Age/ sex	Symptoms	Anamnesis	Identification*	Area	References
1	N/A	N/A	N/A	*D. latum*	Tainan	Frick et al., 1956
2	< 3/F	eggs in stool	N/A	*D. latum*	Taitung	Bergner Jr., 1964
3	> 51/F					
4	8/M	proglottids in stool; gut cramping	eaten raw fish	*D. latum*	Kaohsiung	Chou et al., 2006
5	30/M	proglottids in stool	eaten raw fish	*D. latum*	Taipei	Lou et al., 2007
6	N/A	proglottids in stool	N/A	**1 × *D. latum* (?)**	Taipei	unpublished data (Ro, 2011, MSc thesis)
7				**2 × *D. nihonkaiense***		
8				2 × unidentified species of fish tapeworms		
9						
10						
11	38/M	proglottids in stool	eaten raw fish	**2 × *Dib. nihonkaiensis***	Taipei	present study (2013, 2016)
12	53/M					
13	32/F	proglottids in stool	N/A	*Diphyllobothrium*-like sp.	Taipei	unpublished data (2016)
14	8/F	proglottids in stool	eaten raw fish, but not salmon	***D. latum* (?)**	Taipei	An et al., 2017

N/A – not available; * scientific names originally reported were used; both species now belong to *Dibothriocephalus*; (?) suitable molecular tool was not used; samples analysed genetically in bold.

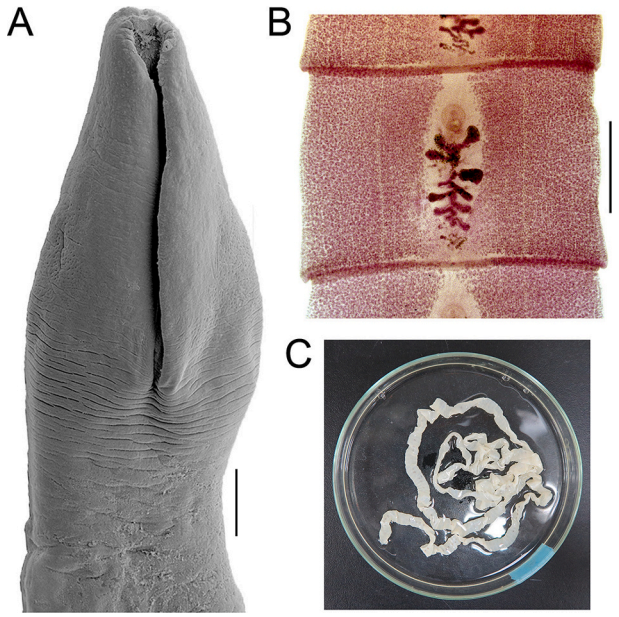

Fig. 1. *Dibothriocephalus nihonkaiensis* from two patients treated in the Taipei Medical University Hospital, Taiwan in 2013 and 2016. Scanning electron micrographs of the anterior part of the body with scolex (A) photomicrograph of gravid (egg bearing) proglottids stained with carmine, ventral view (B), alive tapeworm in saline, released after anthelminthic treatment (C). Scale bars: A – 200 μm, B – 1 mm.

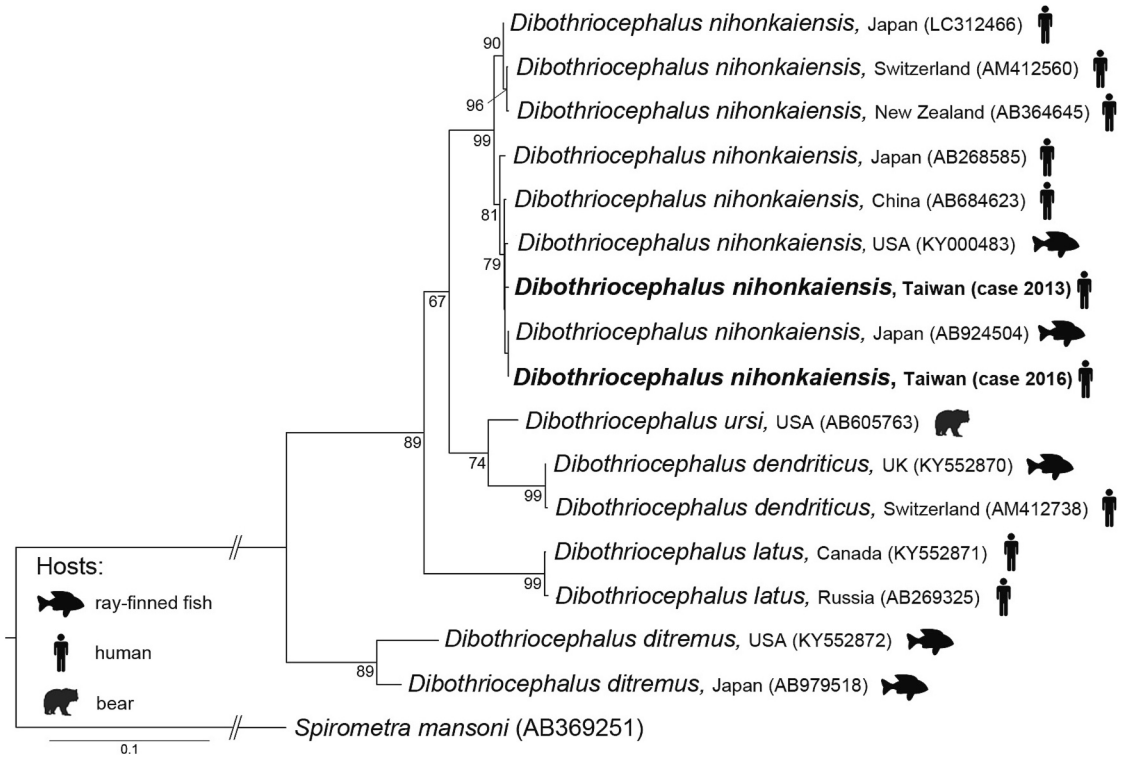

Fig. 2. Molecular identification of two recent human cases of diphyllobothriosis in Taiwan. Phylogenetic relationships represented as a consensus Maximum likelihood tree were calculated using a dataset of fifteen complete cytochrome *c* oxidase subunit I (*cox*1) gene sequences from GenBank and two 513 bp long newly generated sequences using the ultrafast bootstrap approximation in IQ-Tree 2.0.5. Tapeworms acquired from two patients (cases in 2013, acc. no OR625128 and 2016, acc. no. OR625129, in bold) at Taipei Medical University Hospital were identified as *Dibothriocephalus nihonkaiensis* based on high genetic similarity with isolates of *D. nihonkaiensis* from the United States and Japan (pairwise divergence 0–0.2%). An isolate of *Spirometra mansoni* was selected as an outgroup.

Nevertheless, the species identity of the causative agents of human infection in the 1950s and 1960s, when Pacific salmon were not imported into Taiwan, is questionable. The import of infected salmon from the endemic region of *D. nihonkaiensis* with soldiers may be one of the possible explanations. Frick et al. (1956) found eggs identified as those of *D. latus* in one of 1073 Chinese Nationalist soldiers stationed near Tainan. The Chinese soldier retreated from mainland China to Taiwan after the World War II, so he may have contracted fish tapeworm in mainland China. Although Chih-pen is located in the mountains of Taitung, it is an aboriginal community not far from the coast. In the 1960s, many aborigines worked on deep-sea fishing vessels. We suspect that salted Pacific salmon was brought home and shared with family members or neighbours.

The public should be better informed about the risk of eating raw fish, including Pacific salmon, imported unfrozen throughout the world because the larvae (plerocercoids) of broad tapeworms remain infectious to humans when their hosts (fish) are transported on ice (Kuchta et al., 2019). The variety of foodborne parasites that could be spread through globalization presents challenges to clinicians, veterinarians, diagnosticians, and all those involved in food safety (Robertson et al., 2014).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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