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SHORT COMMUNICATION

Molecular identification of the *Diphyllobothrium* species causing diphyllobothriasis in Chilean patients

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Abstract Diphyllobothriasis caused by the infection of adult *Diphyllobothrium* tapeworms sporadically occurs in Chile. The occurrence of the disease is closely linked to the consumption of raw or undercooked freshwater and marine fishes. Diagnosis of diphyllobothriasis has been based on laboratory examinations of the morphological characteristics of proglottids and eggs passed in the feces. Although determination of the parasite to the species level is possible through histologic examination of proglottid specimens, the parasites of patients who only discharge eggs cannot be diagnosed to the species level. Determining the species responsible for the infection of humans and other animals in affected areas is an important component of understanding the epidemiologic and enzootic characteristics of any infectious disease. We therefore compared the classification results obtained using a molecular approach with those obtained from morphological and histopathological examination of proglottids or eggs from five Chilean individuals with diphyllobothriasis. DNA analysis confirmed that the causative *Diphyllobothrium* species in Chile were first identified as *Diphyllobothrium latum* and *Diphyllobothrium pacificum* at least. Furthermore, mitochondrial cytochrome *c* oxidase subunit 1 gene analysis also supported the hypothesis that *D. latum* from Chile originated from Europe.

Introduction

Diphyllobothriasis caused by the infection of adult *Diphyllobothrium* tapeworms is a fish-borne cestodiasis that is endemic to the countries of the southern cone of South America, which includes Chile (Sagua et al. 1976, 2001; Torres et al. 1983, 1993, 1998; Mercado et al. 1988; Kurte

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et al. 1990; González et al. 1999) and Argentina (Semenas and Ubeda 1997; Semenas et al. 2001) and Peru in the Pacific coast (Lumbreras et al. 1982). The occurrence of the disease is closely linked to the consumption of raw or undercooked freshwater or marine fishes. The majority of laboratory diagnoses of diphyllobothriasis have been based on morphological characteristics of expelled proglottids and eggs passed in the feces. Identification of the parasite to species level has depended on the histological examination of proglottids, which means that the parasites responsible for intestinal infections in patients who only discharge eggs could not be identified to species level. However, determining the *Diphyllobothrium* species responsible for human and other animal infections within a particular geographic area is important to understand the epidemiologic and enzootic aspects of diphyllobothriasis. Despite numerous reports on diphyllobothriasis cases in South America, the causative species have never been identified using DNA techniques. In the present study, the results obtained from a molecular diagnosis combined with morphological and histopathological studies of proglottids and eggs obtained from five Chilean individuals with diphyllobothriasis are described.

Case reports

Case 1 The patient was a 3-year-old boy residing in Antofagasta, northern Chile, who complained of proglottid expulsion (Fig. 1a). The patient was likely to have been infected in 2000. Clinical courses were not well-documented, but proglottids were purged naturally and were preserved in

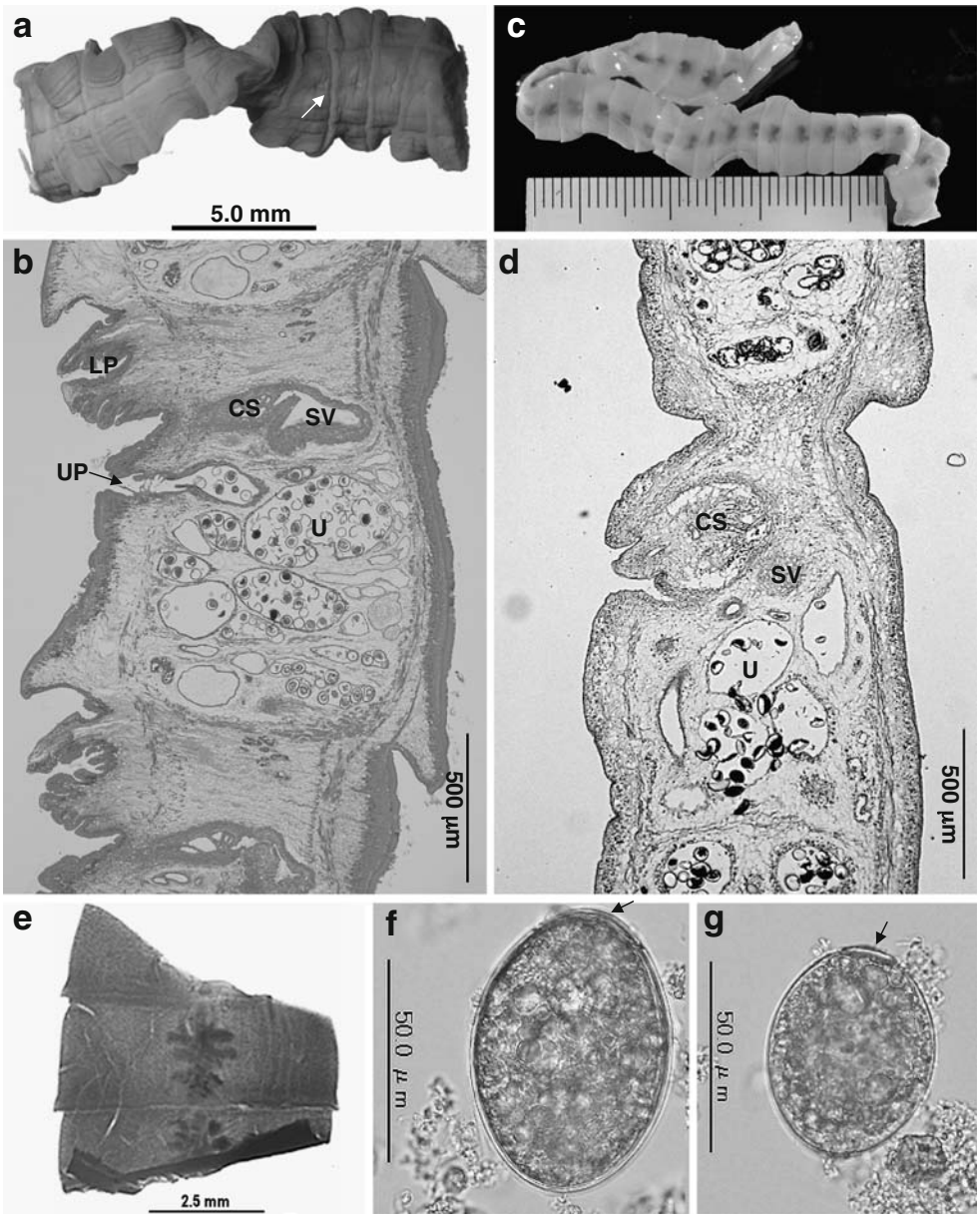

Fig. 1 *Diphyllobothrium* samples examined in the present study. **a** Wrinkled proglottids obtained from Case 1. *Arrow* indicates longitudinal protuberance situated near the uterine pore. **b** *Sagittal view* of the proglottids shown in **a**. *LP* longitudinal protuberance, *CS* cirrus sac, *SV* seminal vesicle, *UP* uterine pore, *U* uterus containing eggs; hematoxylin–eosin stain. **c** proglottids expelled by Case 2. **d** *Sagittal view* of the proglottid shown in **c**; hematoxylin–eosin stain. **e** Proglottids obtained from Case 3. *U* uterus composed of four to six dendritic loops. **f**, **g** Eggs passed in the fecal samples of Cases 4 and 5, respectively. *Arrows* indicate opercula

formalin for 9 years at the University of Antofagasta, Chile. At a retrospective interview, the patient's mother said that they frequently ate a local fish dish, *ceviche*, raw marine fishes, such as corvina (*Sciaena deliciosa*) and cojinova (*Seriolella violacea*) marinated with lemon juice. The child did not have a history of overseas travel.

Case 2 The patient was a 24-year-old man residing in Santiago, the capital of Chile, who expelled proglottids in his feces in 2003 (Fig.1c). The patient stated that he frequently ate salmons from southern Chile, which his mother smoked at home. The tapeworm was successfully purged by administration of praziquantel. The case was also considered to be due to domestic infection because of the lack of overseas travel.

Case 3 The patient was a 13-year-old boy residing in Santiago, Chile. The boy eliminated strobila via the anus (Fig. 1e), which was sent to the Parasitology Laboratory of the Faculty of Medicine, University of Chile in 2009. He frequently ate freshwater fishes, most probably salmonids captured on frequent family fishing trips to the XIV Región de Los Rios in southern Chile. He also ate fried marine fishes. He had no digestive manifestations except for the expulsion of the proglottids when his intestinal contents were evacuated. He was treated with praziquantel and fecal samples were egg-negative in a control examination after treatment.

Case 4 The patient was a 13-year-old girl residing in Santiago, Chile, with no clinical history or epidemiological data. In June 2009, a fecal sample was sent to the Parasitology Laboratory at the Faculty of Medicine at University of Chile, where microscopic examination of the sample showed the presence of abundant *Diphyllobothrium* spp. eggs (Fig. 1f).

Case 5 The patient was a 36-year-old woman residing in Santiago, Chile. She mentioned no trips abroad or to other regions within Chile. A parasitological examination of fecal samples was solicited by her medical doctor after she complained of experiencing digestive discomfort during a routine consultation in March 2009. She mentioned that she frequently ate *ceviche*, as well as salmon, albeit less frequently. Microscopic examination of her feces showed eggs of *Diphyllobothrium* spp. (Fig. 1g). She was treated with praziquantel and microscopic examination revealed that she was egg-negative after treatment.

Materials and methods

The diphyllobothriid samples examined in the present study were obtained as follows: Proglottids (No. 1) from Case 1 and eggs (No. 5) from Case 5 were fixed in 10% and 2% formalin, respectively. Two proglottids (Nos. 2 and 3) from Cases 2 and 3, and an egg sample (No. 4) from Case 4 were preserved in 70% ethanol. For histopathological examination, proglottid samples (Nos. 1 and 2) were processed to paraffin-embedded specimens. The sagittal sections were stained with hematoxylin–eosin and observed.

For the molecular analysis, genomic DNA (gDNA) from the formalin-fixed proglottids (No. 1) was extracted from the 10-μm-thick unstained paraffin sections using a DEXPAT kit (TaKaRa Bio, Japan). For the ethanol-fixed proglottids (Nos. 2 and 3), gDNAs were prepared using a DNeasy Blood and Tissue kit (Qiagen, Germany). For egg samples (Nos. 4 and 5), 10 μL of 50 mM NaOH or 0.1% SDS/proteinase K solution was added to the individual eggs, which were then crushed by physical pressure under a stereomicroscope. The eggs were then lysed in NaOH or SDS/proteinase K at 58~60°C for 1.5 h, and the resulting supernatants were used as template DNA for the polymerase chain reaction (PCR).

Amplification of the mitochondrial cytochrome *c* oxidase subunit 1 genes (*cox1*) was performed by PCR in a 50-μL reaction mixture with *Ex Taq* DNA polymerase (Hot Start version, TaKaRa Bio, Japan). KOD FX DNA polymerase (TOYOBO, Japan) was also used for formalin-fixed samples. To amplify short sequences of the *cox1* genes in formalin-fixed samples (Nos. 1 and 5), the following primer pairs were used: Dpac/F816 (5'-TTTGTTATTCGCCATGTTTTC CATTGT-3') and Dpac/R956 (5'-TTTATACCTGTTGGTA CTCCAATAA-3'), Dpac/F733 (5'-GTGCTTATTTTACCA GGTTTCGGTA-3') and Dpac/R956, and/or Dpac/F920 (5'-CGGTAACCATGATTATTGGAGTACC-3') and Dpac/R1100 (5'-ATATTATCTAACACACAAGCAGAA-3'). For ethanol-fixed samples, the primer pair Diphyllo nad3 (5'-ATGTTAGCTTTATTTTTGGTGG-3') and Diphyllo rrnl/R (5'-ACCAAATAATTGCAATCCTTTCGTAC-3') was used to amplify the complete *cox1* gene. The PCR was performed for 35 cycles of denaturation (94°C, 30 s), annealing (58°C, 30 s), and extension (72°C, 60~90 s) plus one cycle of 72°C for 5 min (Yamasaki and Kuramochi 2009). When KOD FX DNA polymerase was used, the DNA was denatured at 98°C for 15 min prior to perform the PCR described above. Amplicons were confirmed by 3~4% agarose gel electrophoresis or capillary electrophoresis (HAD-GT12, e-Gene Inc., USA) and purified using a NucleoSpin Extract II kit (Macherey-Nagel, Germany) for use as templates for direct sequencing. Samples for direct sequencing were prepared using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Inc., USA) and sequencing was performed on ABI PRISM 3100-*Advant* Genetic Analyzer (Applied Biosystems, USA). Sequence data were analyzed using EditSeq and MegAlign softwares (DNASTAR Inc., USA) and a phylogenetic tree was constructed by the neighbor-joining method using the

MEGA program (version 4.2, http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Results and discussion

Diphyllobothriid samples obtained from five Chilean patients were first identified based on the morphological characters. The surface of the proglottids obtained from Case 1 had a wrinkled appearance and a longitudinal protuberance was observed on the ventral surface (see arrow in Fig. 1a). A sagittal view of the proglottids shows the characteristic longitudinal protuberance, the morphology of the cirrus sac and seminal vesicle with thick walls, and relative position of the cirrus sac and seminal vesicle, all of which are suggestive of *Diphyllobothrium pacificum* (Fig. 1b). The proglottids (Fig. 1c) obtained from Case 2 show the cirrus sac as being obliquely oriented relative to the anterior–posterior axis of the proglottids, with the seminal vesicle located posterior to the cirrus sac (Fig. 1d), all of which are characteristics of *Diphyllobothrium latum*. Although the sagittal views of the proglottids from Case 3 could not be obtained, the proglottids were considered to be from *D. latum* due to the uteri being composed of four to six dendritic loops (Fig. 1e). The eggs passed in the feces of Case 4 appeared operculated, thin-walled, and light yellow–brown in color, with dimensions of 67.8±1.8×48.1±1.0 μm (n=20) and were considered to be typical of *Diphyllobothrium* spp. (Fig. 1f). The eggs found in the feces of Case 5 were elliptic, light yellow–brown in color, with dimensions of 55.7±2.2×42.7±0.9 μm (n=10) and were also considered to be from *Diphyllobothrium* spp. (Fig. 1g). Although the eggs from Case 5 were smaller than the eggs excreted by Case 4, superposition of egg dimensions in *Diphyllobothrium* species has been described (P. Torres, *personal communication*). DNA analysis was performed to confirm the classifications obtained using morphological and histopathological characters.

PCR products containing complete *cox1* gene (approximately 1.7 kb) were amplified in ethanol-fixed samples (Nos. 2, 3, and 4, data not shown). DNA sequencing revealed that the three samples identified morphologically as *D. latum* had identical nucleotide sequences (AB504899, AB510496, and AB511963 for Nos. 2, 3, and 4, respectively) and were confirmed to be *D. latum* by BLAST search and phylogenetic analysis (Fig. 2). Conversely, amplification of the target DNA in formalin-fixed samples (Nos. 1 and 5) was difficult due to DNA degradation by formalin. However, short DNA fragments were successfully amplified and the partial *cox1* sequences of 107 bp (AB525408) and 356 bp (AB517949) obtained from Nos. 1 and 5, respectively, were identified as *D. pacificum* (Fig. 2). *D. pacificum* from Case 1 were not included in Fig. 2 because the gene fragment measured only 107 bp. A transitional nucleotide substitution (G/A) at position 972 of the *cox1* genes was observed between *D. pacificum* isolates from Chile (No. 5) and Peru (AM747494).

Between the time of the first reported human case of diphyllobothriasis in Chile in 1950 (Neghme et al. 1950) and 2001, at least 81 and 16 cases have been attributed to *D. latum* and *D. pacificum*, respectively (Torres et al. 1993; Sagua et al. 2001). In these cases, the etiologic agents were identified based on the morphology of the expelled proglottids and eggs passed in the feces. The present study is the first to confirm the causative species of diphyllobothriasis in South America using a combination of DNA analysis combined with morphological and histopathological examination. Nucleotide sequence of the *cox1* gene of *D. latum* from Chile is identical to those of *cox1* genes of *D. latum* from Europe and Russia (Fig. 2), supporting that *D. latum* in South America was historically introduced

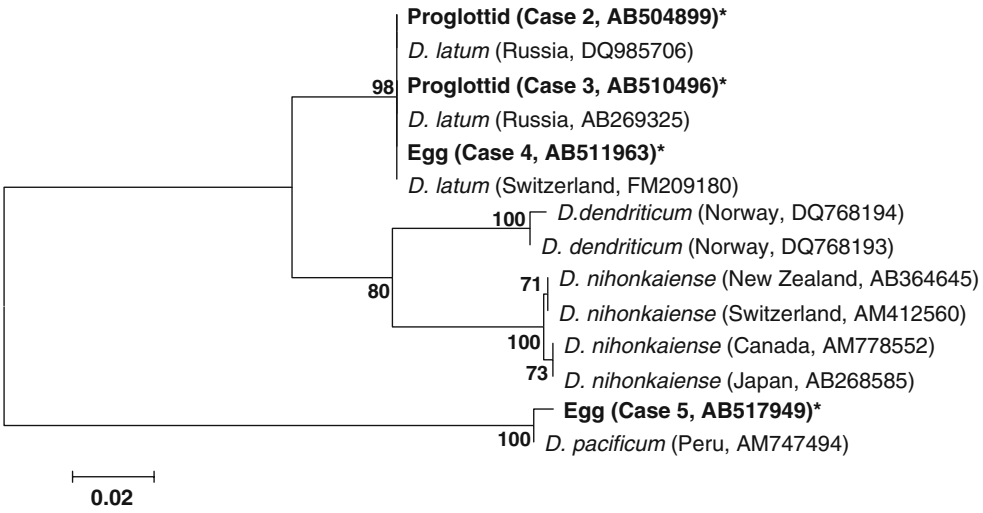

Fig. 2 Phylogenetic tree inferred from *cox1* (396 bp) nucleotide sequences using the neighbor-joining method. Sample No. 5 was based on a 356 bp *cox1* sequence. *Asterisks* indicate *Diphyllobothrium* samples from Chile. *Numbers* at branches denote bootstrap values for 1,000 replicates and the *scale bar* represents the genetic distance based on Kimura's two-parameter model

by European immigrants and tourists infected with the parasite (Neghme et al. 1950; Semenas and Ubeda 1997). To confirm this, further analysis using more samples of *D. latum* from Chile and Argentina is needed.

Patients in Cases 1 and 5 had eaten corvina (*S. deliciosa*) and cojinova (*S. violacea*), both of which have been confirmed as second intermediate hosts of *D. pacificum* in Peru (Escalante and Miranda 1986). In southern Chile, plerocercoids of *Diphyllobothrium latum* and *Diphyllobothrium dendriticum* have been reported to be prevalent in rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*), Atlantic salmon (*Salmo salar*), and brown trout (*Salmo trutta*; Torres et al. 1989a, b, 2000, 2002, 2004; Yamasaki et al. unpublished data); Cases 3 and 4 had histories of eating salmonids. Salmon meat has become an increasingly important global commodity, and increased exports of fresh/chilled salmon have been promoted by rapid advances in international transport systems and technology. Taken together, these advances have resulted in an increase in the emergence of diphyllobothriasis in regions where it was previously absent (Cabello 2007a, b). An outbreak of human diphyllobothriasis in Brazil where it was previously unknown was likely related to the consumption of *sushi* or *sashimi* from aquacultured Atlantic salmon imported from Chile or due to the indigenous common snook (*Centropomus undecimalis*; Sampaio et al. 2005).

Cases 1, 3, and 4 were children. Case 1 was a 3-year-old boy infected by *D. pacificum,* and Cases 3 and 4 were parasitized by *D. latum*. Mercado et al. also reported a *D. pacificum* infection in a 3-year-old Chilean boy (Mercado et al. 1988). These data appear to show that the consumption of fish can cause infections in both children and adults, with none of the apparent age-related bias observed in human-cestode infection cycles associated with the consumption of raw or undercooked bovine and porcine meat (Mercado and Arias 1995).

Given the morphological similarities among diphyllobothriid species, the application of molecular identification methods for accurately identifying *Diphyllobothrium* species is both important and helpful for accumulating geographical, ecological, and epidemiological information related to diphyllobothriid parasites and diphyllobothriasis.

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Nucleotide sequence data reported in the present paper are available in the DDBJ/GenBank/EMBL databases under the accession numbers AB525408, AB504899, AB510496, AB511963, and AB517949 for *Diphyllobothrium* samples Nos. 1, 2, 3, 4, and 5, respectively.