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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

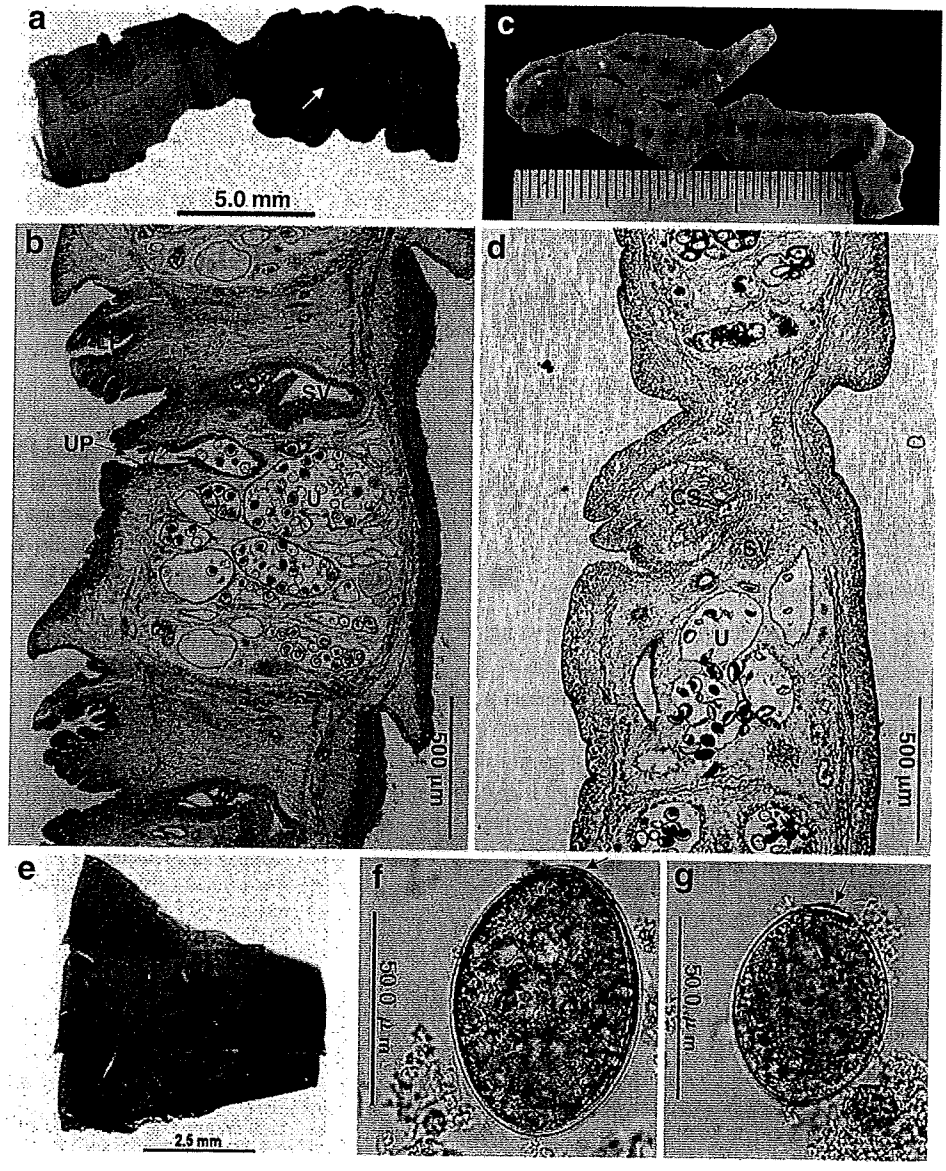

Fig. 1 *Diphyllobothrium* samples examined in the present study. **a** Wrinkled proglottids obtained from Case 1. *Arrow* indicates longitudinal protuberance situated near the uterine pore. **b** *Sagittal view* of the proglottids shown in **a**. *LP* longitudinal protuberance, *CS* cirrus sac, *SV* 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'-ATGTTAGCTTTATTTTTTGGTGG-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

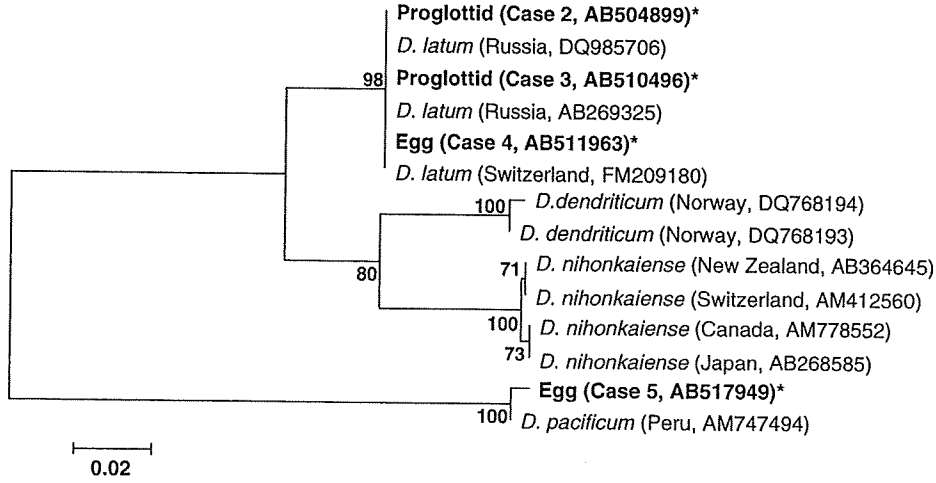

Fig. 2 Phylogenetic tree inferred from *cox1* (396 bp) nucleotide sequences using the neighbor-joining method. Sample No. 5 was based on a 356 bp *cox1* sequence. *Asterisks* indicate *Diphyllobothrium* samples from Chile. *Numbers* at branches denote bootstrap values for 1,000 replicates and the *scale 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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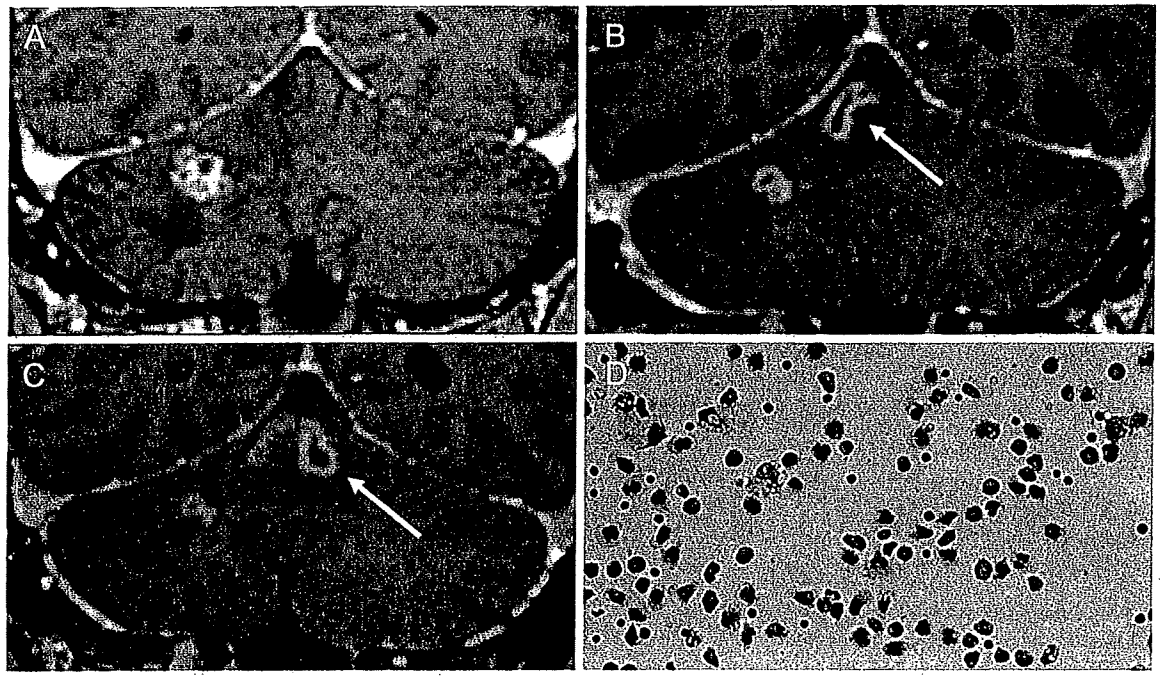
Nucleotide sequence data reported in the present paper are available in the DDBJ/GenBank/EMBL databases under the accession numbers AB525408, AB504899, AB510496, AB511963, and AB517949 for *Diphyllobothrium* samples Nos. 1, 2, 3, 4, and 5, respectively.

Cerebral sparganosis

The wandering lesion

Figure Coronal postcontrast T1-weighted MRI of the same section and CSF cytology



MRIs demonstrate the migration of the lesion from the cerebellar hemisphere to the vermis over a period of 7 weeks. (A) Initial image, (B) 4 weeks later, and (C) 7 weeks later. The tunnel sign appearing as a hollow tube (arrow) represents the moving track of a migrating worm. Giemsa-stained CSF (D) shows a high number of eosinophils.

A 38-year-old Japanese man presented with a 2-year history of cerebellar ataxia, fever, and headaches. MRI revealed migration of the lesion (figure, A–C). CSF cytology showed eosinophilic meningitis (figure, D); serum and CSF ELISA were positive for *Spirometra erinaceieuropaei*.

Human sparganosis is a rare parasitic infection by the larval cestode of *Spirometra* that results from ingesting the plerocercoid harbored in frogs, snakes, and chickens. Reported worldwide, sparganosis is most prevalent in Southeast and Eastern Asia. The diagnosis is suggested by a wandering lesion, especially in endemic areas; the tunnel sign on postcontrast MRI is characteristic.[1] The preferred treatment is the surgical removal of live worm.

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Disclosure: Dr. Shirakawa and Dr. Yamasaki report no disclosures. Dr. Ito serves as an Associate Editor of *PLos Neglected Tropical Diseases* and on the editorial boards of *Parasitology* and *Parasitology International*. Dr. Miyajima reports no disclosures.

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南米チリよりDNA鑑別依頼のあった裂頭条虫について

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Key Words：裂頭条虫症，太平洋裂頭条虫，広節裂頭条虫，チリ，DNA解析

はじめに

Diphyllobothrium 属条虫の成虫寄生によるヒトの裂頭条虫症は欧州，極東ロシア，東アジア（日本，韓国），北米や南米で発生が見られる。南米チリでは，広節裂頭条虫（*Diphyllobothrium latum*）と太平洋裂頭条虫（*Diphyllobothrium pacificum*）がヒトに寄生する種として知られ，1974年以来，報告された症例数は広節裂頭条虫によるものが81例[1]，太平洋裂頭条虫によるものが16例に及ぶ[2]。しかしながら，原因となった裂頭条虫種が遺伝子解析によって同定されることはなかった。今回，われわれは2000年と2004年にチリで発生した裂頭条虫症2例について，チリ大学より虫体のDNA解析依頼があったのでその結果を報告する。

症　例

症例1

患者はチリ北部のアントファガスタに住む3歳のチリ人男児で，2000年に発症し，駆虫によって得られた片節はチリ大学医学部で約9年間ホルマリンに浸漬されていた。患者の母親によると，海産魚のイシモチに似たcorvinaやcojinovaを材料にしたチリの伝統的な生魚料理cevicheを習慣的に食べてい

Two Human Cases with Diphyllobothriasis in Chile : Molecular Confirmation of the Causative Tapeworms

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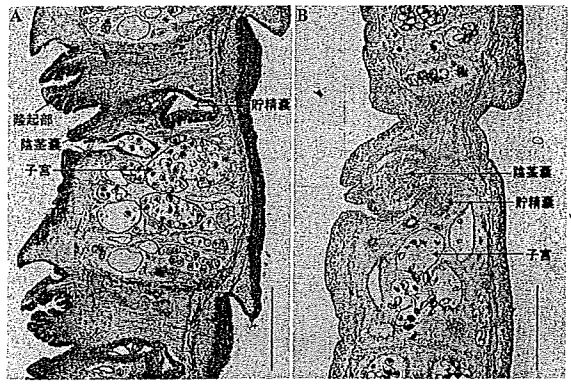

図1　裂頭条虫片節の正中矢状断組織像.
A，症例1の虫体；B，症例2の虫体.
Scale bars = 500 μm.

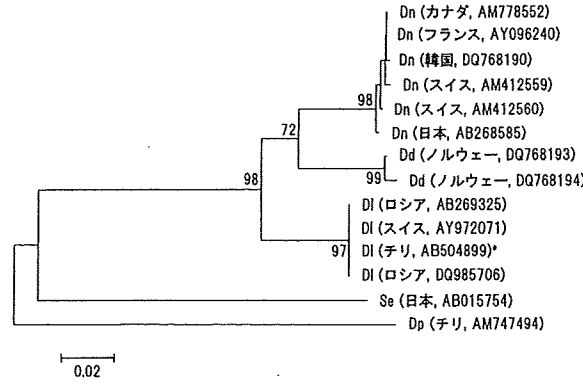

図2　*cox1* 遺伝子断片（396bp）の塩基配列に基づいた症例2の虫体の同定．系統樹は近隣結合法によって作成した.
Dn, D. nihonkaiense（日本海裂頭条虫）；***Dd, D. dendriticum***；***Dl, D. latum***（広節裂頭条虫）；***Se, Spirometra erinaceieuropaei***（マンソン裂頭条虫）；***Dp, D. pacificum***（太平洋裂頭条虫）．*，症例2の虫体．括弧内は産地と accession numbers．分岐部の数字は1000反復による bootstrap 値.

たという。チリ以外への渡航歴はなく，チリ国内感染と考えられた。駆虫された虫体は黄褐色で，ホルマリン固定による強い収縮のためか片節は肉厚で，片節幅は4～5mm，片節表面には皺や皺が多く，片節前縁から生殖孔にかけての正中線上に隆起部があり，片節の正中矢状断組織像でも隆起部が確認された（図1A）。陰茎嚢は片節の前後軸に対して斜位を取り，貯精嚢は長楕円形で壁が厚く，陰茎嚢の下壁背端に接続していたことから，太平洋裂頭条虫と考えられた。

症例2

患者はチリの首都，サンチアゴ在住の24歳のチリ人男性で，2004年に発症し，駆虫によって得られた片節はエタノール固定され，チリ大学医学部で保存されていた。患者はチリ産の養殖サケ・マスの自家製燻製をよく食べていたという。渡航歴はなく，チリ国内での感染と考えられた。虫体は白色で，片節幅は約1cm，片節中央部に虫卵で充満した子宮が観察された。片節の正中矢状断切片像では，陰茎嚢は楕円球形で片節の前後軸に対してやや斜めに位置し，貯精嚢はそれより小さく，楕円球形を呈し，陰茎嚢の後方に位置していた（図1B）。

DNA 解析

症例1，2の虫体とも，DNAは市販のキット（DNAeasy Tissue/Blood kit, Qiagen）を用いて抽出し，ホルマリン固定された症例1の虫体は18S rRNA遺伝子と28S rRNA遺伝子の短い断片（250bp）を標的に，一方，エタノール固定された症例2の虫体はミトコンドリアゲノムでコードされるcytochrome *c* oxidase subunit 1遺伝子の全長（1,566 bp）をPCRによって増幅し，その増幅産物の塩基配列を解析した[3]。その結果，症例1の虫体はホルマリン固定によるDNA分解のために，種鑑別ができる領域の増幅が十分でなかったが，症例2の虫体は広節裂頭条虫と同定された（DDBJ/GenBank accession number AB504899，図2）。

考　察

チリでは，広節裂頭条虫と太平洋裂頭条虫の2種がヒトに寄生する種として報告されているが，いずれの症例においても片節や虫卵の形態に基づいて原因種の同定がなされてきた[1)2)]。症例1の虫体はその形態と太平洋裂頭条虫の第2中間宿主として知られる海産のニベ科の魚，corvina（*Sciaena deliciosa*）やcojinova（*Seriolella violacea*）[4]の摂取歴から太平洋裂頭条虫と考えられたが，増幅された18S，28S rRNA遺伝子の短い断片では種の同定には至らず，現在ミトコンドリアDNAの解析を試みている。症例2の虫体は，陰茎嚢が片節の前後軸に対して水平位を取るとされる広節裂頭条虫の特徴は観察されな

かったが，遺伝子解析によって広節裂頭条虫と確定された。

チリでは，ニジマス（*Oncorhynchus mykiss*），ギンザケ（*Oncorhynchus kisutch*）やブラウントラウト（*Salmo trutta*）の養殖がさかんであり，養殖ニジマスの他，土着の淡水魚 Patagonian smallmouth perch（*Percichthys trucha*）や puye（*Galaxias maculates*）において広節裂頭条虫や *Diphyllobothrium dendriticum* のプレロセルコイドが高率で寄生していることが報告されており[5)6)]，患者はチリ産の養殖サケ・マスの自家製燻製の摂取歴があった。

最近では，チリ産サケ・マスが感染源と推定される広節裂頭条虫症が今まで知られていなかったブラジルで報告されたことにより[7)8)]，チリ以外の国における裂頭条虫症の発生が懸念されており[9)]，わが国もチリ産養殖サケ・マスを輸入していることから，裂頭条虫症の原因種を形態のみならずDNA解析によって正確に鑑別することは裂頭条虫症の感染経路や予防に関する疫学的情報を集積するという意味で重要と考えられた。

謝　辞：裂頭条虫の形態について有益な助言をいただいた目黒寄生虫館　荒木潤先生，ならびに国立科学博物館動物研究部の倉持利明先生に深謝する。本研究は2008年度厚生労働科学研究費補助金新興・再興感染症研究事業「顧みられない病気に関する研究，H20-新興一般016」の助成によって行われた。

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皮膚病診療：31(8)；977～980, 2009

◇虫による皮膚病－2009<臨床例>――⑯

皮膚二核顎口虫症

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Key words
遊走性皮疹，顎口虫症，二核顎口虫，ペルー

・顎口虫症は，野生哺乳動物を終宿主とする顎口虫の第3期後期幼虫が非好適宿主であるヒトに摂取され，人体内を移動することによりさまざまな症状を呈する幼虫移行症である．
・ペルーで生魚料理セビッチェ(ceviche)を食べた後に，遊走性皮疹が出現し，皮膚生検組織内虫体の遺伝子解析により二核顎口虫症と確定診断した1例を経験したので報告する．

症例　44歳，男．海外出張が多い．

初診　2007年10月．

主訴　右下肢の遊走性皮疹．

家族歴，既往歴　特記すべきことはない．

現病歴　2007年9月下旬から10月初旬にペルーへ渡航し，現地の郷土料理であるセビッチェという生魚のマリネを食べた．帰国して数日後に咽頭痛，頭痛が出現した．10月下旬に右膝上方に腫脹，発赤，熱感，軽度の圧痛が出現したため，その2日後に当院整形外科を受診した．白血球とCRPの軽度の上昇（WBC 9,690/μl，Eos 5.6%，CRP 0.75mg/dl）より，蜂窩織炎と診断され，塩酸セフカペンピボキシル(フロモックス)，ロキソプロフェンナトリウム(ロキソニン)にて加療されるも，皮疹が右大腿へ移動してきたため，整形外科受診後8日目に当科を紹介受診した．

当科初診時現症　右大腿前面上方に，熱感・圧痛を伴う限局性の腫脹・発赤を認めた．

臨床検査成績(下線部は異常値)

WBC 9,020/μl(Neut 55.0%，Eos 11.0%)，RBC 550×10^4/μl，Hb 15.9g/dl，Plt 30.0×10^4/μl，AST 35 IU/l，ALT 48 IU/l，T-Bil 0.49mg/dl，BUN 14.0mg/dl，Cr 0.94mg/dl，CRP 0.18mg/dl．

鑑別診断

臨床的に遊走性皮疹を呈し，末梢血好酸球の上昇が認められたこと，さらに海外渡航先での魚の生食歴があったことから，皮膚遊走性限局性腫脹をきたす寄生虫疾患を考えた．移動性皮膚病変を呈するものとして，線状疹では顎口虫症，旋尾線虫症や動物由来の鉤虫症，限局性腫脹ではマンソン孤虫症，イヌ糸状虫症，肺吸虫症などが知られている[1]．そこで，国立感染症研究所寄生動物部でドロレス顎口虫，イヌ糸状虫，マンソン孤虫，ウェステルマン肺吸虫，宮崎肺吸虫の5種類の寄生虫抗原を用いてELISA法を行ったところ，ドロレス顎口虫とイヌ糸状虫抗原に対して陽性反応であった．

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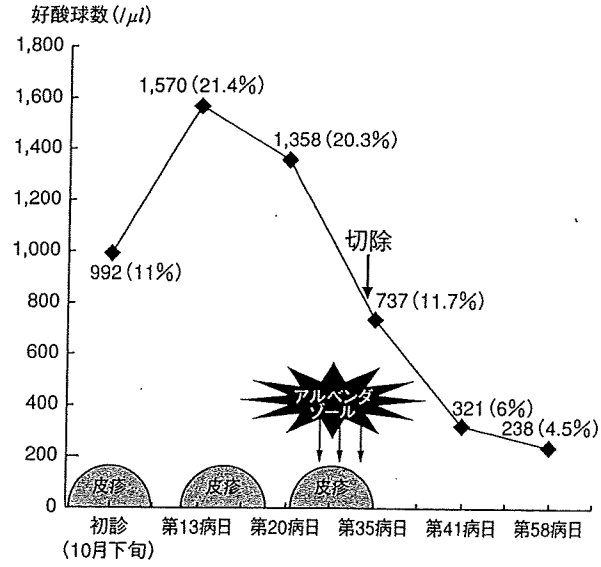

図1　臨床経過．皮疹は約1カ月にわたり消褪と再発を繰り返し移動した．好酸球は21.4%にまで上昇を認めたが，アルベンダゾール投与後に，皮疹を切除した後は順調に低下し，正常化した．

旋尾線虫：ホタルイカの生食が感染源であることから否定された．

イヌ糸状虫症：イヌ糸状虫の幼虫に感染したカがヒトを吸血し感染する．臨床症状として咳，痰，胸痛，発熱などが出現する．イヌ糸状虫抗原に対する陽性反応は，イヌ糸状虫と同じ線虫類の粗抗原を用いたことによる交叉反応であると考えられた．

マンソン孤虫症：幼虫であるプロセルコイドが寄生するケンミジンコの摂取，あるいはプレロセルコイドが寄生するヘビ，カエル，地鶏の生食により感染する[2]．臨床症状としては，不規則な発熱や移動性の皮下腫瘤が出現するが，移動速度は遅く，自験例では摂食歴もなく，血清診断結果も陰性であった．

肺吸虫症：サワガニなど淡水産カニに寄生した肺吸虫メタセルカリアを摂取した場合，あるいはサワガニを食したイノシシ筋肉内に寄生した肺吸虫を摂取した場合に感染し，咳・胸痛，血痰，胸水貯留などの症状が出現するほか，皮下腫瘤を形成することもあるが，自験例では摂食歴，臨床症状，血清検査から否定された．

診断，治療と経過

皮膚の発赤，熱感を伴う限局性腫脹は，10月下旬には右膝上方に，その10日後には右大腿上方の前面に，さらにその約2週後にはその後面に，11月下旬には右膝上方の内側に，と約1カ月にわたり消褪と再発を繰り返し移動した．その間，好酸球は21.4%にまで上昇を認めた(図1)．血清診断ELISA法にてドロレス顎口虫抗原に陽性反応を示したことより顎口虫症と考え，アルベンダゾール(エスカゾール)400mg/日を，11月下旬の右膝上方内側の腫脹出現時より3日間投与したところ，内服を開始してから7日目に，皮疹は右下肢腓腹部に移動し再発を認めた．それまで限局性の腫脹を呈していた皮疹は，右下肢腓腹部では線状爬行疹を呈していた(図2)．

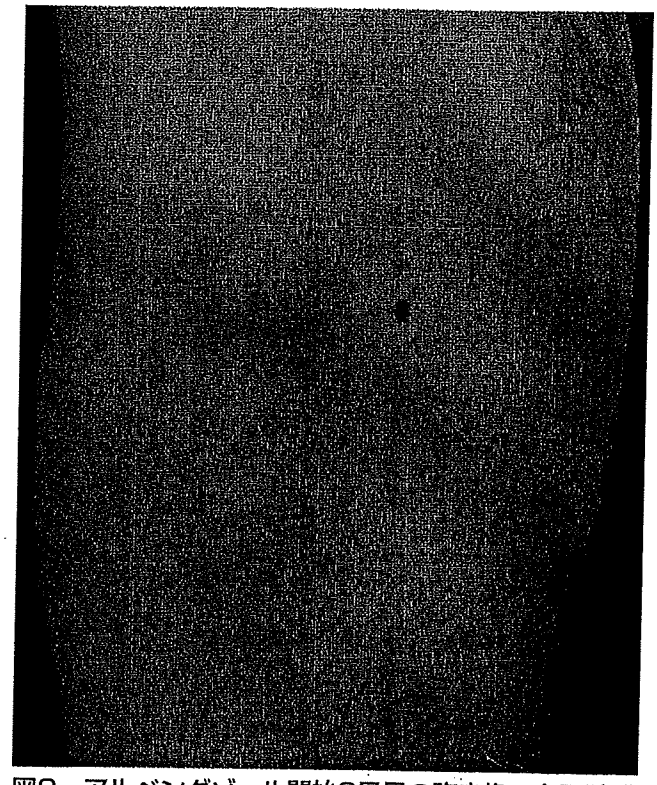

図2　アルベンダゾール開始8日目の臨床像．右下肢腓腹部に線状爬行疹がみられたため，両端を切除した．

皮疹の進行方向が不明であったが，その両端部のいずれかに顎口虫がいると考え，診断確定と治療を兼ね，12月初旬に皮疹の両端部を切除した．線状爬行疹の上方の先端部より生検した標本の真皮中層に虫体を確認でき，虫体周囲には好酸球，リンパ球を主体とする炎症細胞の浸潤を認め，虫体表面には多数の皮棘が密生し，体腔内に大きく突き出した双葉状の側索が観察され，顎口虫幼虫

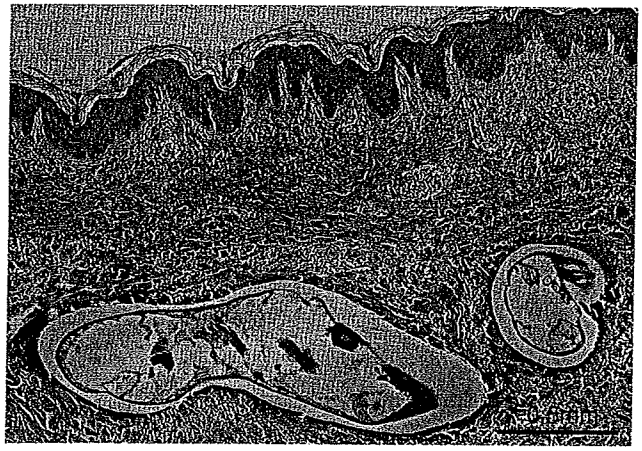

図3　真皮中層に虫体の断面を認め，その周囲には好酸球，リンパ球を中心とする炎症細胞浸潤を認める(H-E染色，×40)．

と同定された(図3)．

そこで，顎口虫の種の鑑別を行うために，国立感染症研究所寄生動物部にて，パラフィン包埋無染色切片内の虫体断端標本を用いて，ミトコンドリアDNAのcytochrome *c* oxidase subunit 1遺伝子(*cox1*)とリボソームRNA遺伝子内のITS-2領域の塩基配列を解析した．その結果，既知の二核顎口虫(*Gnathostoma binucleatum*)の塩基配列と100%一致したことから，本症例を二核顎口虫症と診断した．自験例のDNAデータは以下のアクセッション番号で登録されている(*cox1*はAB374229とAB428417，ITS-2領域はAB428418)．

虫体を摘出した後は，皮膚症状の再燃はなく，好酸球数も順調に低下し12月上旬には正常化した．

考　按

現在，世界には12種の顎口虫が知られており[3,4](表)，人体寄生の報告があるのは，有棘顎口虫，ドロレス顎口虫，日本顎口虫，剛棘顎口虫，二核顎口虫の5種である[3]．本邦では，第二次世界大戦直後の食糧事情が悪かった時期に，雷魚の生食による有棘顎口虫の感染が西日本で流行した．1980年ごろからは，中国，韓国や台湾産の輸入ドジョウの生食による剛棘顎口虫の感染が西日本の大都市を中心に流行した．東北地方では国産ドジョウや，ブラックバスの生食による日本顎口虫の感染が，九州全域，四国，中国地方では渓流魚の生食によるドロレス顎口虫の感染が報告されている[1,3]．

顎口虫成虫は終宿主である哺乳類の胃壁や食道などの消化管粘膜に寄生しており，終宿主の糞便とともに排出された虫卵は水中で発育，孵化し第2期幼虫となり，これが第1中間宿主であるケンミジンコに取り込まれ，その体内で第3期前期幼虫になる．ケンミジンコがさらに第2中間宿主(魚類，両生類)に摂取されると，その体内で第3期後期幼虫となる．ヒトは第3期後期幼虫が寄生した第2中間宿主(魚類，爬虫類)を摂取することで，感染する．

ヒトが感染すると，前駆症状として微熱，感冒様症状，倦怠感，食欲不振などを呈する．またヒトに経口摂取された幼虫は，腸管から一度腹腔に出て肝臓を通過するので肝障害が出現することもある[5]．皮膚症状は感染の約3週間後より出現し，線状爬行疹や限局性腫脹を呈する[3]．皮疹は痒みや軽度の疼痛を伴いながら移動し，数カ月～数年間持続する．そのほか，歩行・運動障害，痙攣，意識障害，気胸，イレウス，腹膜炎，心筋梗塞，眼科・耳鼻科・泌尿器科的障害など，さまざまな症状を呈する[6]．

遊走性皮疹などの臨床所見，魚類，両生類などの生食歴，海外居住歴・海外渡航歴などの詳細な問診，末梢血好酸球数や血清IgE値の上昇等から本疾患を疑い，皮内反応やELISA法による免疫学的血清反応を行う．末梢血好酸球数や血清IgE値は，感染期間や個体により差異がみられる．本疾患患者におけるドロレス顎口虫抗原を用いた皮内反応では，陽性率82.4%，偽陽性16.6%，またELISA法では陽性率93.7%，偽陽性27.7%で，感度は高いが，特異性はやや低いとする調査結果もある[7]．免疫学的血清反応は，各種線虫類抗原間で交叉反応がみられ，また検査方法や評価が各検査機関で一定していないなどの欠点があり，あくまで補助診断であり，診断確定には治療を兼ねて虫体を摘出する方法が考えられるが，虫体検出はむずかしい．虫体が摘出された場合には，頭球鉤の形態や数，腸管上皮細胞の形態や核数の観察により顎口虫の種の同定が可能であるが[8]，虫体断端によっては，これらの形態が観察されるとは限らないので，自験例のように遺伝子解析が種の同定に有用である．

表　顎口虫属の独立種(赤羽，岩田〈2005〉を改変)

学名，和名	終宿主	寄生部	分布地
G. spinigerum Owen, 1836　有棘顎口虫	ネコ科，イヌ科	胃壁	アジア，オセアニア
G. hispidum Fedtschenko, 1872　剛棘顎口虫	ブタ，野生ブタ	胃壁	アジア，ヨーロッパ
G. turgidum Stossich, 1902　オポッサム顎口虫	オポッサム	胃壁	アメリカ大陸
G. americanum Travassos, 1925　アメリカ顎口虫	ネコ科	胃壁	南米
G. doloresi Tubangui, 1925　ドロレス顎口虫	ブタ，イノシシ	胃壁	アジア，オセアニア
G. nipponicum Yamaguti, 1941　日本顎口虫	イタチ	食道壁	日本
G. procyonis Chandler, 1942　アライグマ顎口虫	アライグマ	胃壁	アメリカ大陸
G. miyazakii Anderson, 1964　宮崎顎口虫	カワウソ	腎臓	アメリカ大陸
G. malaysiae Miyazaki et Dunn, 1965　マレーシア顎口虫	ネズミ	胃壁	マレーシア，タイ国
G. vietnamicum Le-Van-Hoa, 1965　ベトナム顎口虫	カワウソ	腎臓	ベトナム，タイ国
G. binucleatum Almeyda-Artigas, 1991　二核顎口虫	ネコ科，イヌ科	胃壁	メキシコ以南のアメリカ大陸
G. lamothei Bertoni-Ruiz et al, 2005　和名なし	アライグマ	胃壁	メキシコ

治療は，虫体の摘出がもっとも確実だが，虫体の移動速度が速く[9]，深部皮下組織に迷入することもあること，などから，皮疹部のみを生検しても虫体が摘出できないことも多い．皮疹の進行部先端のいまだ正常にみえる皮膚をできるかぎり大きく切除することが推奨されている[10]．駆虫剤としてアルベンダゾールが使用されることが多いが[1,6,8-12]，単独では奏効率が低い．駆虫剤の内服により虫体の移動速度が下がり，また虫体が皮膚浅層に遊走してくる傾向があるので，摘出に成功する可能性が高くなる[12]．

経過・予後は種によって異なるが，皮膚症状は数カ月～数年間持続する例が多く，中枢神経系に迷入すれば致命的となることもある．

自験例の原因種となった二核顎口虫は，1991年にメキシコで山猫から成虫が発見され[13]，ヒトへの感染源としてテラピアなどの淡水魚の生食が指摘されている．二核顎口虫の形態は，有棘顎口虫と類似しているが，腸管上皮細胞内の核数が，二核顎口虫では平均2個であるのに対し，有棘顎口虫では平均3～4個と報告されているが[8]，自験例では確認できなかった．ペルーでの顎口虫症感染例はこれまでに2例報告されているが[8,14]，いずれも種の同定には至っておらず，自験例は病理組織標本を用いた遺伝子解析により二核顎口虫症と確定診断された本邦初の報告例，世界でも2番目の報告例である．

近年，海外渡航者数の増加に伴い，自験例のように邦人が海外で感染し，帰国後国内で発症する，いわゆる輸入寄生虫症例の報告が相次いでいることから，われわれ皮膚科医も寄生虫疾患の可能性を念頭に置いて診断する必要があると考えられた．

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Ectopic (Subcutaneous) *Paragonimus miyazakii* Infection in a Dog

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Abstract. Ectopic infection with *Paragonimus miyazakii* was determined to be the cause of a subcutaneous inguinal mass in a 15-month-old, male, boar-hunting dog. On histologic examination, the mass comprised granulomatous panniculitis, intralesional adult trematodes and eggs, and lymphadenitis. Extrapulmonary paragonimosis in animals is rare. This appears to be the first report in a dog of ectopic *P. miyazakii* infection with mature trematodes and eggs that involved the inguinofemoral lymphocenter and surrounding subcutis.

Key words: Dogs; ectopic parasitism; histopathology; lymphadenitis; *Paragonimus miyazakii*; subcutis.

Paragonimosis is a parasitic disease caused by trematodes of the genus *Paragonimus* and is an important food-borne endemic zoonosis worldwide.[6,8] Adult trematodes reside in the lungs of definitive hosts (humans and various wild and domestic animals, including dogs).[6,8] Ectopic paragonimosis with larvae and/or adults is well recognized in humans;[3,6,8] however, only a few cases of natural or experimentally induced ectopic paragonimosis were reported in dogs.[1,2,5,7,14] To the best of our knowledge, this represents the first report of extrapulmonary *P. miyazakii* infection in a dog with mature trematodes and eggs in an inguinal mass that involved lymph nodes and surrounding subcutis.

A 15-month-old, setter-type, male dog that had been used for boar hunting was presented to a private animal hospital for examination of a palpable inguinal mass. Reportedly, the dog occasionally ate raw wild boar meat. Lymphoma was suspected clinically, so a core biopsy of the inguinal mass was performed. After unidentifiable fragments of a parasite were detected on histologic examination, excisional biopsy was performed for diagnosis and therapy.

On gross examination, the formalin-fixed subcutaneous mass was gray and lobulated, approximately 16 × 10 × 10 cm. The cut surface of the mass was mottled yellow-brown to dark-red and contained trematodes and enlarged lymph nodes. By stereomicroscopy, the trematodes, encapsulated in cysts, were whitish, had a thick ellipsoidal body and a small, reddish, crater-shaped acetabulum. Eggs were operculate, irregularly barrel shaped, and averaged 77.7 μm × 48.0 in length and width.

On histologic examination, the subcutaneous mass comprised granulomatous panniculitis with cysts that contained adult trematodes and eggs, and lymphadenitis associated with the eggs. Dense, broad bands of fibrous tissue encapsulated the mass and dissected between the cysts and nodules (Figs. 1, 2). Cysts contained single or paired adults, scattered mature eggs, mixed inflammatory cells, red blood cells, and necrotic debris. Nodules consisted of numerous mature and degenerated eggs in granulomatous inflammation (Fig. 2). Some nodules contained a central mass of eggs and a thin peripheral rim of fibrosis; in others, solid nodules of eggs were separated by fine fibrous septa. Infiltrating leukocytes included a variable number and mixture of plasma cells, lymphocytes, neutrophils, eosinophils, and macrophages, with a few multinucleated giant cells. The affected lymph nodes had both small and massive clusters of eggs scattered throughout the nodal sinuses (Fig. 3). Some egg clusters elicited granulomatous response. Follicles were hyperplastic with prominent germinal centers; nodal fibrosis was severe and diffuse.

On histologic examination, the adult trematodes had tegument, with a single spine, well-developed oral and ventral suckers, uterus, ovary, and intestine. Numerous mature eggs were in the uterus; lobes of the ovary were moderately branched. Testes and vitelline ducts were also fully developed and contained mature sperm and vitelline cells, respectively.

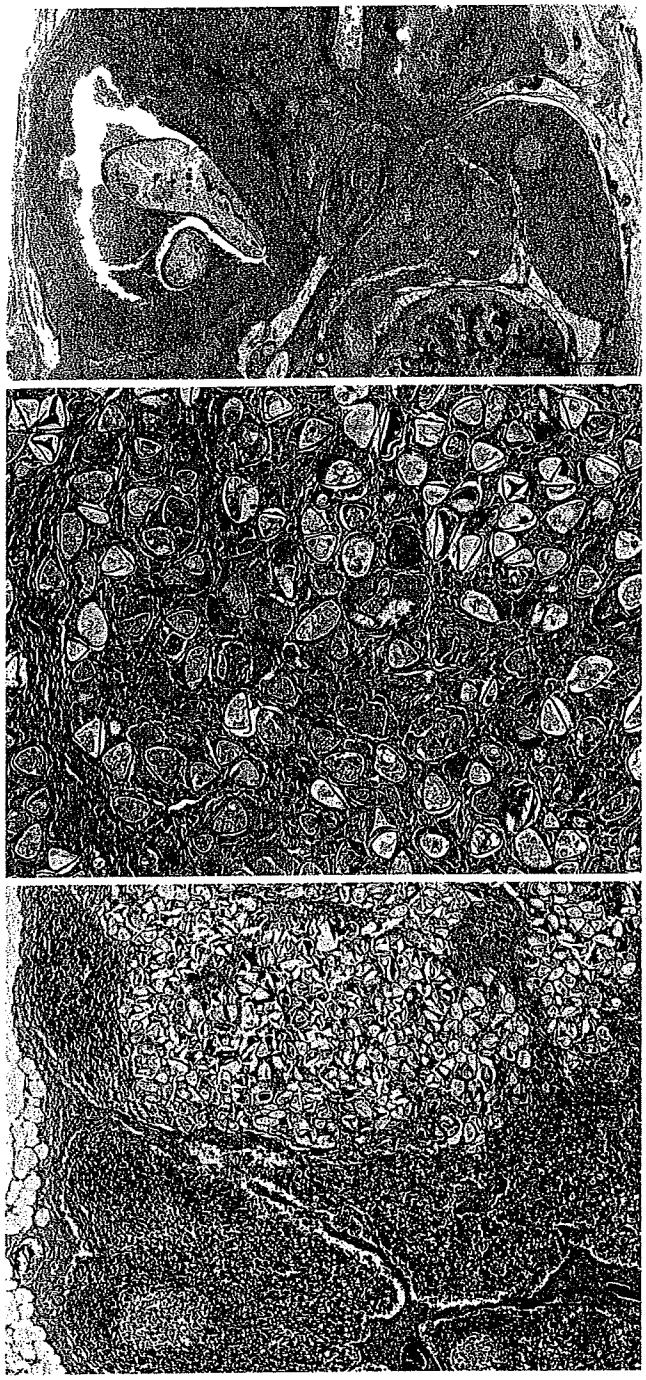

Fig. 1. Inguinal subcutaneous mass; dog. Granulomatous panniculitis and lymphadenitis associated with adult trematodes and eggs. HE. Bar = 1 mm.

Fig. 2. Inguinal subcutaneous mass; dog. Clusters of trematode eggs in granulomatous inflammation. HE. Bar = 100 μm.

Fig. 3. Lymph node of the inguinofemoral lymphocenter; dog. Granulomatous lymphadenitis with trematode eggs. Lymphoid follicles have prominent germinal centers. HE. Bar = 400 μm.

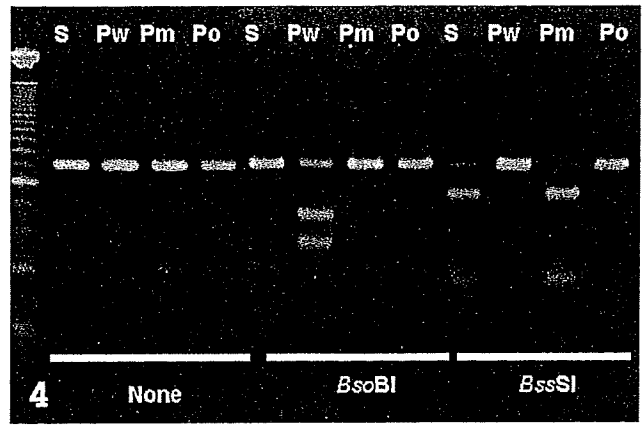

Fig. 4. RFLP analysis of ITS2 PCR products. The DNA from the sample (S), *P. westermani* (diploid type) (Pw), *P. miyazakii* (Pm), and *Paragonimus ohirai* (Po) was amplified with ITS2-specific PCR primers and digested with endonucleases, *Bso*BI, and *Bss*SI. The restricted fragments of the sample are identical to those for *P. miyazakii*.

Microscopic features of the adult parasites and stereomicroscopic features of the eggs were characteristic of *Paragonimus* spp., so ectopic paragonimosis was diagnosed. After approximately 2 weeks, the excision site swelled again to approximately two thirds of its size before surgery. Oral praziquantel treatment (10 mg/kg, once a day, for 10 days) was administered. The swelling subsided after the treatment, and the dog has remained free of clinical disease, without further treatment, for more than 2 years. Additional clinical examinations, including chest radiography or fecal examination for *Paragonimus* eggs, were not performed.

The *Paragonimus* sp. was identified from the formalin-fixed specimen by polymerase chain reaction (PCR) linked restriction fragment length polymorphisms (RFLPs) and DNA sequencing.[12] Restricted fragment length polymorphisms of the second internal transcribed spacer (ITS2) from nuclear ribosomal DNA were identical to those of *P. miyazakii* (Fig. 4), and the sequences of ITS2 were identical to those deposited in the GenBank/EMBL/DDBJ nucleotide database (accession number U96912) for *P. miyazakii*. The species was thus identified as *P. miyazakii*.

Paragonimosis is an important food-borne parasitic zoonosis caused by trematodes (genus *Paragonimus*), which infect the lungs of humans and various other animals.[6,8] At least 28 species of *Paragonimus* have been identified,[8] and 10 species are recognized as causing human disease.[6] *Paragonimus* spp. are mainly parasites of cats, dogs, and various mammals that eat freshwater crabs and crayfish (the second intermediate host), as well as the raw meat of wild boars (the paratenic host).[9] Occasionally, humans become accidental hosts.[8] With approximately 200 million people at risk and 22 million people infected worldwide, paragonimosis still is an important public health threat.[6] In the Far East,

including Japan, the prevalence of human parasitic diseases has been greatly reduced as living standards improved, but pulmonary paragonimosis remains an important endemic parasitic disease.[11] Dogs and cats, as more likely definitive hosts, generally play a greater role than humans in the *Paragonimus* life cycle.[6]

In veterinary medicine, 2 *Paragonimus* spp., *Paragonimus westermani* and *Paragonimus kellicotti*, are of particular interest.[4] *P. westermani* is the best-known species in Asia; *P. kellicotti* occurs in North America. In addition, in Japan, *P. miyazakii* is another important species responsible for human and animal paragonimosis.[3,6,10]

Paragonimus trematodes usually infect the lungs of the mammalian host.[3] However, aberrant migration may be more likely in accidental hosts,[8] and, because humans are less suitable than other mammals as a definitive host for *Paragonimus*, they may be more commonly affected by ectopic paragonimosis. In human extrapulmonary paragonimosis, various sites and tissues, including the brain, spinal cord, abdominal cavity, or subcutis, may be involved;[3,6,8] pathologic effects are principally caused by the presence of adult trematodes and eggs, the movement of trematodes through tissues, and the metabolites produced by trematodes.[3] Some species differences exist in the frequency and effect of ectopic paragonimosis. Subcutaneous masses associated with parasitic migration reportedly occur in 20–60% of *Paragonimus skrjahbini* infections, compared with approximately 10% with *P. westermani* and 2.4% with *P. miyazakii* (2/82).[6,10] For those *Paragonimus* spp., such as *P. miyazakii* and *P. skrjahbini*, for which humans are an unsuitable host, the trematodes are unlikely to mature, so eggs are rarely found in extrapulmonary sites.[3]

In dogs, ectopic paragonimosis is rare.[1,2,5,7,14] In reported cases, extrapulmonary lesions were mostly produced by migration of immature trematodes or by eggs;[5,14] exceptional cases had *P. westermani* cysts and eggs in trachobronchial lymph nodes[7] or spleen.[2,5] In contrast, the present case developed an inguinal subcutaneous mass that contained encysted solitary or pairs of adult trematodes. The trematodes had matured in this ectopic location, forming cysts and producing eggs. Granulomatous panniculitis and lymphadenitis were mainly attributed to the presence of eggs. Because dogs are definitive hosts for *P. miyazakii*, the incidence of canine extrapulmonary paragonimosis should be low. However, once *P. miyazakii* settles in an extrapulmonary site, maturation and egg laying may occur, as in the present case.

In this dog, the trematodes may have migrated to an inguinal lymph node, mated, deposited eggs, and provoked lymphadenitis and regional panniculitis. In human medicine, lymphadenitis because of the presence of metazoan parasites, including *Paragonimus* or their eggs, is rare but has been described.[13] In dogs, such lymphadenitis is also rare but has been described with *P. westermani*[7] and *P. kellicotti* infection.[1] However, lesions in those cases were much less severe than in the present case.

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Detection of *Paragonimus* Metacercariae in the Japanese Freshwater Crab, *Geothelphusa dehaani*, Bought at Retail Fish Markets in Japan

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Metacercariae, the encysted larval stage of flukes capable of infecting the final and/or paratenic hosts, of *Paragonimus miyazakii* and of both diploid and triploid forms of *P. westermani* are found in the Japanese freshwater crab, *Geothelphusa dehaani*, which acts as the second intermediate host in Japan. This crab is known as Sawagani in Japanese and is widely distributed in Japan, from Hokkaido to Kyushu islands, including Yakushima Island. Both *Paragonimus* spp. are known to be medically important causes of human infection, although the respiratory symptoms that develop in patients vary according to the form and species of the causative lung fluke. Chronic cough with rusty-colored sputum is the most common symptom of patients infected with the triploid form of *P. westermani*, while infection with *P. miyazakii* and the diploid form of *P. westermani* usually causes pleural effusion without remarkable lesions in the lung parenchyma (1).

In Japan, the incidence of *Paragonimus* infection has increased among long-term foreign residents (2,3). It is postulated that long-term residents from Asian countries such as China, Korea and Thailand maintain their dietary habits in Japan and, thus, ingest uncooked Sawagani in their ethnic dishes. Infection of people outside of these groups who eat these dishes has also been reported. There is a need for caution regarding paragonimiasis associated with these eating habits. In some cases, the causative foodstuff included in these dishes was identified as Sawagani sold at local retail fish markets.

In the present study, we purchased Sawagani originating from three prefectures (Shizuoka Prefecture in the Tokai district, and Miyazaki and Nagasaki prefectures in the Kyushu district) at retail fish markets in the Tokyo metropolitan area between April 2004 and February 2008 and examined these crabs for the prevalence of *Paragonimus* metacercariae (Table 1). Lung fluke metacercariae were detected in 44 (17%) of 266 examined crabs. The positive crabs harbored a total of 169 metacercariae, with the average numbers of metacercariae being 3.8 and 0.64 per positive crab and per crab of the total number of crabs examined, respectively. The maximum number of metacercariae in a single crab was 23 in a crab originating in Miyazaki Prefecture that was purchased in February 2008.

Individual metacercariae isolated from the crabs were identified to the species (*P. westermani* or *P. miyazakii*) and, further, to the form (diploid or triploid) for *P. westermani*. The metacercariae of *P. miyazakii* could be morphologically discriminated from those of *P. westermani* by the presence of a membranous substance, as well as by the absence of a stylet (1). Of a total of 169 isolated metacercariae, both of these characteristics were confirmed in only 20 metacercariae, which were identified as *P. miyazakii*. The remaining metacercariae were subjected to molecular identification by PCR-restriction fragment length polymorphism (RFLP) analysis and sequencing. First, the total genomic DNA was prepared from individual metacercariae following our previously described method (4). The ITS2 region of the nuclear ribosomal DNA (rDNA) and a portion of the 16S mitochondrial rDNA were amplified by PCR using primer pairs 3S (forward: 5′-GGTACCGGTGGATCACTCGGCTCGTG-3′) with A28 (reverse: 5′-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3′) (5) and T7-1 (forward: 5′-ATTTACATCAGTGGGCCGTC-3′) with SP6-1 (reverse: 5′-GATCCAAAAGCATGTGAAAC-3′) (6), respectively. The amplified products were treated with restriction enzymes and separated by electrophoresis on agarose gel (RFLP analysis). For the RFLP analyses, we selected restriction enzymes *Sna*BI and *Bss*SI to digest the ITS2 PCR products from *P. westermani* and *P. miyazakii* (4). We selected enzymes *Sna*BI and *Bsr*DI based on the theoretical restriction maps generated from the 16S mitochondrial rDNA sequences of diploid and triploid forms of *P. westermani* (6,7). Undigested amplicons were sequenced using the corresponding primers to verify the identification made by RFLP analy-

Table 1. Prevalence, number and species of *Paragonimus* metacercariae in Japanese freshwater crabs, *Geothelphusa dehaani*, sold at retail fish markets in the Tokyo metropolitan area, Japan

Month of purchase	Origin (Prefecture)	No. of crabs examined	No. of crabs infected	No. of Mc[1)] detected	Species[2)] of Mc
Apr. 2004	Shizuoka	48	0	0	
Apr. 2007	Miyazaki	46	0	0	
Apr. 2007	Miyazaki	16	7	29	Pm
Apr. 2007	Nagasaki	21	5	9	Pm
June 2007	Shizuoka	35	0	0	
June 2007	Miyazaki	44	5	9	Pw (3n)
Jan. 2008	Miyazaki	30	4	6	Pm, Pw (2n)
Feb. 2008	Miyazaki	26	23	116	Pm
Total		266	44	169	

[1)]: Metacercariae.
[2)]: Pm, *P. miyazakii*; Pw (2n), the diploid form of *P. westermani*; Pw (3n), the triploid form of *P. westermani*.

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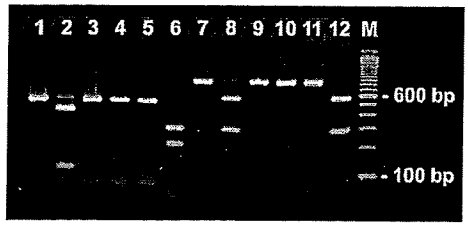

Fig. 1. RFLP patterns of PCR products amplified from the DNA of *P. westermani* metacercariae (lanes 1-3 for both the diploid and triploid forms; lanes 7-9 for the diploid form; lanes 10-12 for the triploid form) or *P. miyazakii* metacercariae (lanes 4-6). The ITS2 PCR products were untreated (lanes 1 and 4) or treated with endonucleases *Sna*BI (lanes 2 and 5) or *Bss*SI (lanes 3 and 6). The 16S rDNA PCR products were also untreated (lanes 7 and 10) or treated with endonucleases *Sna*BI (lanes 8 and 11) or *Bsr*DI (lanes 9 and 12). A 100-bp DNA ladder marker was used to estimate the size of the fragments.

sis.

PCR amplification with the primer pair 3S and A28 generated single 520-bp products from the metacercarial DNA samples. Electrophoresis of the restriction enzyme-digested products resulted in two species-specific RFLP patterns, as previously described (4). Species identification of the metacercariae was made based on the digestion patterns of amplification products. Products that were digested with *Sna*BI to produce 2 fragments (about 420 bp and 100 bp) but remained undigested with *Bss*SI were identified as those of *P. westermani*. Products that were undigested with *Sna*BI but were digested with *Bss*SI to produce 2 fragments (about 300 bp and 220 bp; Fig. 1) were identified as those of *P. miyazakii*.

DNA samples prepared from *P. westermani* metacercariae were further analyzed to determine the form, i.e., diploid or triploid. PCR amplification of mitochondrial DNA with the primer pair SP6-1 and T7-1 produced a single 840-bp product. Restriction digestion of PCR products was used to identify the diploid and triploid forms. Products that were digested with *Sna*BI to produce 2 fragments (about 550 bp and 290 bp) but remained undigested with *Bsr*DI were identified as those of the diploid form. Products that remained undigested with *Sna*BI but were digested with *Bsr*DI to produce 2 fragments (about 560 bp and 280 bp; Fig. 1) were identified as those of the triploid form. The species and forms identified by the RFLP analyses were verified by sequencing of the respective PCR products.

Consequently, as shown in Table 1, most of the metacercariae were identified as *P. miyazakii* (157 metacercariae from 36 positive crabs), while the others were *P. westermani* (3 metacercariae from 3 positive crabs and 9 metacercariae from 5 positive crabs were of the diploid and triploid forms, respectively). However, there were no mixed infections either with *P. miyazakii* and *P. westermani* (diploid and/or triploid forms) or with both forms of *P. westermani* in any crab examined in the present study.

Sawagani from Miyazaki Prefecture were also purchased at a retail fish market in Fukuoka City in April 2008 and were examined for *Paragonimus* metacercariae. *P. miyazakii* metacercariae (35 in total) were detected in 15 of 30 examined crabs. This finding implies that Sawagani with *Paragonimus* metacercariae that are responsible for human infections are likely also sold in retail fish markets in areas other than Tokyo.

The heat resistance of *P. westermani* metacercariae within the crab hosts was investigated almost a century ago (8). The Japanese mitten crab, *Eriocheir japonicus*, which played a major role as the second intermediate host in spreading the human infection of *P. westermani* at that time in Japan was investigated (*P. miyazakii* metacercariae have never been isolated from this crab species). It was shown that boiling infected crabs at 55°C for 5 min killed all the metacercariae (8). However, to the best of our knowledge, the conditions required to kill metacercariae of *P. westermani* and *P. miyazakii* in Sawagani have not yet been well examined, although we are currently investigating these conditions. Therefore, the implementation of a health education campaign is recommended throughout Japan to emphasize that Sawagani, even those sold at retail fish markets, are potential sources of lung fluke infection in humans. Special attention should be paid to ethnic dishes that are prepared with uncooked Sawagani.

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