れたシラウオの調査(1972～1973年)では北海道産シラウオからは全く検出されず，本州産では3～8割が陽性，特に霞ヶ浦産の感染率が高い[10]。1999年における霞ヶ浦の調査でも，この感染率の高さが確認されている。シラウオの平均寄生メタセルカリア数はほぼ20以下であるが，最高314虫体寄生例も報告されている[11]。その後の調査では，霞ヶ浦でも特に北浦のシラウオの感染率が高い事が示されている[12, 13]。

ヒトだけでなく，魚を捕食したり，拾って食べるような哺乳類(主に肉食獣)や鳥類も終宿主となる。患者糞便由来の虫卵がカワニナに伝播する機会は，わが国ではまれと予想される。イヌおよびネコは個体数が多く，ヒトより一般に感染率が高い。さらに，キツネやタヌキでの感染率も高い[5]。したがって，特に高橋吸虫および宮田吸虫の生活環はこれらのペットや野生動物と，カワニナ，淡水・汽水魚間で主に伝播しているものと考えられる。

終宿主における成虫の発育は早く，感染後10日ほどで産卵を開始し[1]，1年間ほど寄生する。小腸に寄生する *Metagonimus* spp. 成虫の病原性はほとんどなく，通常臨床症状は示さない[5]。

5 動物のメタゴニムス症

終宿主における特異性は低い。わが国において，イヌ，ネコ，キツネ，タヌキ，ハクビシン，アライグマ，テン，イタチ，ブタ，ドブネズミ，カワネズミ，トビ，カモメ，トラツグミ，ゴイサギなどから成虫が検出されている[14]。実験感染ではさらに，ハムスター，コットンラット，マウス，ラット，ウサギ，ニワトリなどにおいても成虫に発育する[1]。3種の *Metagonimus* の宿主域はほぼ同様と考えられる。

成虫の寄生期間はマウスやニワトリのように短いものでは1カ月以下のものもあるが，平均1年前後のものが多い。実験感染におけるマウスとイヌの比較では，イヌでは横川吸虫の発育が早く，虫体も大きいことが知られており，動物種において感受性に差がある[1]。さらに，同一宿主種においても顕著な系統差があることがマウスで示されている[15]。イヌでは感染後1～1.5カ月でEPG (eggs per gram) は最高に達し[16]，その後虫卵の排泄が続き，長期観察では2年以上の寄生期間であった。マウスおよびイヌでは部分的な再感染防御が報告され，再感染時は定着する虫体数が少なく，寄生期間も短くなる[15]。

最も多く剖検調査されている動物はイヌであり，1970年以降のイヌについての多くの調査では *Metagonimus* spp. は検出されなかったが，その他の調査では1.2～6.9%から検出されている[17]。例外的に1979年，米田らは福岡・佐賀における54頭の調査で，約半数から検出している。ネコの剖検調査でも多くの調査において検出できなかったが，その他の調査では1.8～14.7%から検出されている[17]。寄生虫体数は多いもので数百である。感染率は一般に幼獣よりも成獣で高い。高橋吸虫と横川吸虫を区別している調査では，ほとんど高橋吸虫と同定されている。近年のイヌ，ネコの感染率は低い。たとえば，埼玉県の1999～2007年のイヌ905頭，ネコ1,079頭の直腸便を用いた糞便検査で，*Metagonimus* は1頭のネコからのみ発見されている[18]。

野生動物の剖検では，タヌキの2～13.5%から検出されている[19]。キツネについては北海道での調査が多く，7.9～93%陽性となっており，青森，秋田では11%の感染率である。千歳市周辺のアライグマの調査では，22%陽性(1999年，27/124未発表)となっている。これらの野生肉食獣からは，*Metagonimus* 3種とも検出されている。さらに，北海道のトビでは36%（5/14)から検出され，寄生虫体数も多く *Metagonimus* 流行におけるトビの重要性も示唆されている[1]。

概して，横川吸虫や宮田吸虫は小腸上部に，高橋吸虫はやや下部に寄生する傾向があるが，混在している症例も多い。通常，成虫は小腸の絨毛間に寄生するが，ネコやイヌでは虫体はまれに腸陰窩に寄生する場合があり[20, 21]，免疫抑制時にはより深く侵入することがマウスで示唆されている[22]。

Metagonimus の病原性は弱く，通常は無症状である。ネコおよびイヌの重度感染ではカタール性腸炎となり[5]，小腸の浮腫，軽度の炎症，絨毛の短縮と陰窩の深長，杯細胞の減少が認められ[20, 21, 23]，さらに，マウス，ラットでは肥満細胞増加と小腸の粘膜透過性の亢

進[24]，末梢血および小腸中部における軽度の好酸球増加[25]および小腸粘膜上皮における刷子縁の酵素活性の変化が報告されている[26]。横川吸虫は，宮田吸虫よりも病原性が少し強いことが示唆されている[27]。

本症の診断は糞便検査による。ネコでは感染後1週よりIgG抗体がELISA法で検出されるようになるが[28]，診断には利用されていない。

予防のためには，イヌやネコに淡水・汽水魚を生で与えないことが肝心である。流行地においてはペットに魚を給餌する場合は，十分加熱後与え，さらにイヌは係留し，魚を拾い食いさせないようにする。また，流行地のネコについては室内飼育，もしくは係留しないかぎり，完全な予防は困難である。

治療は，プラジクアンテル30 mg/kg経口，皮下もしくは筋肉内投与1回で著効を奏する[29]。

6 ヒトのメタゴニムス症

国内ではヒトにおいて最も感染率の高い寄生虫で，なんらかの寄生虫に感染している患者の半分以上がメタゴニムス症である。*Metagonimus* spp. のヒトの感染率は，虫卵の糞便検査（近縁種の異形吸虫の虫卵も含む）による日本寄生虫予防会による全国調査において，1970～1990年代に増加し(0.01～0.67%)，近年(2004～2008年）では減少傾向（0.05～0.01%）が報告されている。しかし，ある関東の病院における1998年の人間ドック2,235例の検査では188例（8.4%）から虫卵を検出し[30]，2001～2004年の海外赴任予定者（3,914人）を対象とした検査では69例（1.7%）が陽性と報告されている[31]。検査の対象者によっても感染率にかなり差がある。1992～1997年における韓国全土での住民の*Metagonimus*感染率は0.3%と報告され，河川沿岸においては40%と高い地域もある[32]。

ヒトのメタゴニムス症はほとんど横川吸虫によるとされてきたが，大型の虫卵，すなわち高橋吸虫と思われる虫卵を排泄した症例も多く報告されている[10]。また，ヒトにおける宮田吸虫の感染も日本・韓国で報告されているが[4]，どの程度横川吸虫と混同しているかの判別は今後の課題である。

わが国では主にアユ，シラウオ，ウグイなどの魚の生食もしくは加熱不十分な料理によりヒトへ感染する。したがって，このような食事の頻度が感染の機会を決める。たとえば，アユの解禁後，ヒトの感染率が急増する。韓国でもアユ，オイカワ，コイがヒトの主たる感染源となっている。ヒトにおける感染期間は長いものでは4年近く寄生していたという報告がある。寄生虫体数は多くの場合1,000以下であるが，多いものでは5万虫体以上検出された例もある[32, 33]。

感染の機会が多いと考えられる河川湖沼沿岸の住民を対象とした，ほぼ日本全国的な糞便検査による調査において[8]，北日本での感染率は低いが，南日本は8.7～73.9%陽性と高くなっており，特にアユ漁の盛んな河川湖沼沿岸では高く，71.8～73.9%という報告もある[1]。1973年，影井・木畑は，アユのメタセルカリア寄生虫体数と住民の虫卵陽性率に相関があり，アユのメタセルカリア虫体数からその地域住民における流行をある程度予測できるとしている。シラウオもしばしば生食に供され，霞ヶ浦周辺の患者はシラウオが主たる感染源であり，宮田吸虫によるメタゴニムス症と推察される[1]。関東の某病院の検査結果では，1991～1999年までメタゴニムス虫卵陽性率が上昇し，この時期には関東地方のシラウオの漁獲量が2倍に増えているが，宮田吸虫かどうかは確認されていない。

*Metagonimus*成虫の病原性は低く，自覚症状はほとんどない。虫卵陽性者と陰性者のアンケート調査では，胃部不快感，下痢，嘔吐，腹痛を訴えるものが虫卵陽性者に多い。重度感染でも自覚症状のあるものとないものがある[32]。臨床検査でも血液検査でも異常は認められず，重度感染時に好酸球増多が時折認められる程度である。

感染から虫卵が糞便に排泄されるまでの期間（プレパテントピリオド）は10日と短く，本症の診断は糞便検査（特に，ホルマリン・エーテル法もしくはAMS Ⅲ法〔Army Medical School Ⅲ method〕）で虫卵を検出し，虫卵の形態をもとに行う。小型の虫卵で他の異形吸虫との形態的な鑑別は困難であるが（高橋吸虫は少し大きい），胆管に寄生する肝吸虫との鑑別は可能である。

以前は，駆虫薬としてカマラやビチオノールがしば

しば用いられたが33)，現在ではプラジクアンテルが推奨されている。韓国では 20 mg/kg 1 回投与では駆虫効果 88%，2 回投与では 100%と報告されている32)。2002 年，原島らも 109 例について 1 回投与で 97.6%陰転，残り（3 例）は 2 回目に陰転したと述べている30)。

7 公衆衛生，経済，社会へのインパクト

様々な淡水・汽水魚にメタセルカリアの寄生が認められ，動物へはヒトの食用に供されない魚種も重要な感染源となる。消費者へ安全な食品を提供するという立場からは，アユとシラウオにおけるメタセルカリア感染率の高さは非常に問題である。

近年，若干の減少傾向があるものの，ほぼ一定して患者の発生があり，今後とも患者の発生が予想される。*Metagonimus* の病原性はほとんどないため，糞便検査によってはじめて本寄生虫に感染していることが判明する。このように，感染を知るためには住民の糞便検査の必要があるが，重度感染は極めてまれであり，流行地であっても住民の定期診断は行われていない。主要な生活環のサイクルが動物間で行われていることから，住民の診断・治療を行ったとしても，メタゴニムス症の制圧は困難である。動物間の生活環を断ち切ることは極めて困難である。すなわち，カワニナ，第 2 中間宿主となる様々な魚，もしくは終宿主となる野生動物を淘汰もしくは駆虫することは不可能である。

アユを－3℃の緩慢冷凍 3 日間で感染を防げることが報告されている1)。ヒトへの感染予防だけを考慮すると，魚の冷凍によるメタセルカリア殺滅が理論上可能であるが，市場から食品として供されるアユやシラウオ全量を冷凍後消費者に提供することは，現在の日本人の好みから受け入れられないと予想される。さらに，これは市場を介さずに消費者が入手する魚については対応できない。

8 発生と制圧対策

各地域による天然アユおよびシラウオのメタセルカリア感染率も含め，生食による感染の危険性が高いことを販売者，消費者および地域住民に啓蒙することが重要と考えられる。しかし，このような病原性の低い寄生虫予防のため，日本人の食習慣を改めることは容易ではない。霞ヶ浦産シラウオについては，茨城県と東京都との協議により，2000 年から関係団体が自主的に「加熱調理用」表示を行っている12)。

アユの養魚場において，カワニナもしくはカワニナに由来するセルカリア侵入を防げれば，感染していないアユの生産が可能であり，メタセルカリアのいないアユの養魚場もある9)。ちなみに，アユの河川における漁獲量は 1991 年をピーク（約 17,000 t）に減少し，2008 年には 3,456 t となっている。養殖アユも減少傾向ではあるが 2008 年には 5,864 t で，天然物の 1.7 倍量となっている。

一方，シラウオの漁獲量はアユと比べると明らかに少なく，352 t であるが（農林水産統計），しばしば生食に供されるため，ヒトへのメタゴニムスの感染源としては重要と考えられる。天然アユ・シラウオともに漁獲量が年々減少してきているが，感染率の高い水域のシラウオについては，「加熱調理用」表示を徹底していくことが，住民の予防のために必要である12, 13)。

（奥祐三郎）

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Short communication

Gene silencing in *Echinococcus multilocularis* protoscoleces using RNA interference

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ABSTRACT

We investigated the potential of gene silencing in *Echinococcus multilocularis* protoscoleces using RNA interference (RNAi). For the introduction of siRNA, soaking and electroporation were first examined for their effects on the viability of protoscoleces and their efficacy for siRNA introduction. Consequently, electroporation using 100 V and 800 μF showed the optimal results. This electroporation procedure was then evaluated for its ability to induce RNAi in protoscoleces using siRNAs targeting the *14-3-3* and *elp* genes. It was found that the levels of *14-3-3* and *elp* mRNA in *14-3-3* siRNA- and *elp* siRNA-treated protoscoleces were reduced to 21.8 ± 2.6 and 35.5 ± 0.4% of those of the untreated control by day 3, respectively. Moreover, the target proteins significantly decreased in the siRNA-treated samples by day 15. In the analysis of viability, the untreated control, electroporation control, *14-3-3* siRNA-treated, and *elp* siRNA-treated samples displayed 98.4 ± 1.4, 83.0 ± 2.5, 58.0 ± 23.0, and 55.1 ± 14.6% viability, respectively, on day 15. In conclusion, we successfully demonstrated that RNAi mediated the knock-down of target gene expression in *E. multilocularis* protoscoleces at both the transcriptional and translational levels.

Larval stage infection with *Echinococcus multilocularis* causes alveolar echinococcosis, one of the most lethal helminthic infections in humans, demonstrating a greater than 90% fatality rate in untreated patients [1]. Recently, *E. multilocularis* has emerged as a model experimental research system given its interesting characteristics, including its continuous active proliferation and underlying molecular mechanisms of host-parasite interactions [2], in addition to the availability of well-developed *in vivo* maintenance methods using mice and gerbils and *in vitro* cultivation systems [3]. Studies in this organism are also facilitated by available genetic information, such as cDNA sequences and genomic databases (i.e., http://fullmal.hgc.jp/em/; http://www.sanger.ac.uk/Projects/Echinococcus/), long-term *in vitro* cultivation techniques of primary cells, and the ability to transiently transfect cells using plasmids and *Listeria monocytogenes* [4]. However, no methods have been established so far, that allow to knock-out or knock-down the expression of specific genes in *E. multilocularis*.

In this study, we investigated the potential of gene silencing in *E. multilocularis* protoscoleces using RNA interference (RNAi). RNAi is a mechanism in which the degradation of mRNA is induced by complementary short interfering RNA (siRNA) through intracellular mechanisms that have been widely conserved during evolution; as a consequence of these events, gene expression can be effectively suppressed [5]. An advantage of RNAi is its ability to provide information regarding gene function in a relatively quick and easy manner [6], and represents a promising technique for gene function analysis that has been successfully applied in numerous organisms to date. In the phylum Platyhelminthes, RNAi was achieved via soaking and electroporation processes for the introduction of siRNA in schistosomules [7–9], *Fasciola hepatica* [10], and planaria [11]. Among cestodes, Pierson et al. recently reported successful RNAi in adult worms of *Moniezia expansa* using dsRNA [12]. It was therefore expected that this technique could also be applied to *E. multilocularis*, which would greatly facilitate the investigation of gene function and identification of essential gene products of this deadly parasite, and should help to provide important knowledge for the control of echinococcosis.

The protoscoleces of *E. multilocularis* can be isolated in large quantities from fertile metacestode tissues obtained from laboratory animals, and can subsequently be easily maintained *in vitro* for several weeks. Interestingly, protoscoleces are able to develop into either adults or larval vesicles [13]. Thus, protoscoleces represent an attractive research material to study the mechanisms and factors involved in several developmental stages of this parasite.

The *14-3-3* (GenBank accession no. U63643) and *elp* (AJ012663) genes of *E. multilocularis*, which encode for 14-3-3 and antigen II/3, respectively, served as the model target genes in this study. The 14-3-3 proteins constitute a family of conserved ubiquitous eukaryotic proteins that are involved in a number of important cellular processes, including signal transduction, cell-cycle control, apoptosis, stress response, and malignant transformation [14]. In *E. multilocularis*, one 14-3-3 isoform has been published so far which is similar to the zeta isoform in higher eukaryotes and is highly expressed at the metacestode stage and mainly localizes to the germinal layer of cysts

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and the apical structures in both invaginated and evaginated protoscoleces [15]. The protein encoded by the *elp* gene, which has been referred to as antigen II/3, Em10, or Em18, is expressed in both *E. multilocularis* adult and metacestode stages and has been used as an important diagnostic antigen [16]. Antigen II/3 shares homology with the mammalian ezrin, radixin, and moesin (ERM) protein family [17] that is involved in several key processes related to cellular architecture maintenance, including cell–cell adhesion, membrane trafficking, microvillus formation, transmembrane signaling, and cell division [18]. In *E. multilocularis*, antigen II/3 localizes within the germinal layer and parenchymal cells of protoscoleces and on the surface of calcareous corpuscles (Fig. 1 of [19]). Matsumoto et al. reported that the combination of 14-3-3 and antigen II/3 expression could be used as a molecular marker of viability and growth activity in *E. multilocularis* [20].

All experiments in this study were performed using protoscoleces isolated from the *E. multilocularis* Nemuro strain maintained at the Hokkaido Institute of Public Health (Sapporo, Japan). To isolate protoscoleces, larval cyst masses isolated from cotton rats (*Sigmodon hispidus*) were minced, passed through a sieve (100-μm mesh size), and then washed repeatedly with 0.85% physiological saline until host materials were thoroughly removed. The protoscoleces were then treated with 0.5% hydrochloric acid and 0.5% pepsin dissolved in 0.85% physiological saline for 30 min to digest any remaining host tissue (this treatment can be a stimulus for differentiation to adults), and were subsequently cultured in CMRL1066 medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 200 U/ml penicillin G, and 200 μg/ml streptomycin (Gibco) in culture flasks for 1–2 days at 37 °C in the presence of 100% N_2.

For the RNAi experiments, we attempted to select suitable conditions in which protoscoleces could maintain high viability. The negative control RNAi experiments were performed using fluorescently labeled (FAM-labeled negative control #1 siRNA, Ambion) and negative control (Silencer negative control #1 siRNA, Ambion) siRNAs. The sequence of negative control #1 siRNA was unknown, as it is proprietary information of Ambion; however, the company states that the siRNA does not specifically target any human, mouse, or rat gene. To determine the suitable conditions for siRNA introduction, soaking and electroporation were tested without siRNA. Soaking was performed with 1–30 μl transfection reagent (NeoFX, Ambion) in 100 μl culture medium and electroporation was performed at 100–400 V (100-V intervals) and 100–1,000 μF (100-μF intervals) to examine their effects on the viability of the protoscoleces. The results of these tests revealed that protoscoleces exhibited greater than 80% viability 3 days after either soaking with 0–10 μl transfection reagent or electroporation at 100 V with 100–800 μF or 200 V with 100 μF.

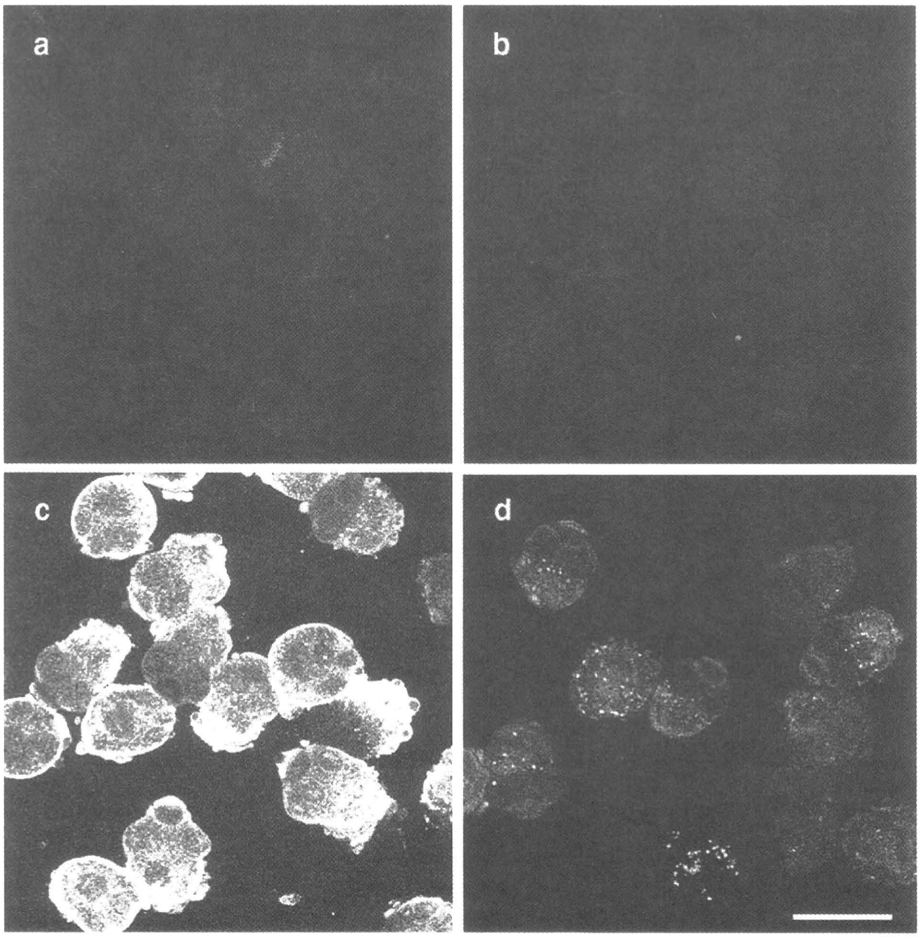

Fig. 1. Localization of fluorescently labeled siRNA following soaking or electroporation. Parasites untreated (a) or treated with fluorescently labeled siRNA via soaking in transfection reagent (b) or electroporation (c and d) were observed by confocal microscopy. At 30 min after electroporation, strong fluorescence was detected in the parasites (c). Diminished fluorescence and several bright spots were observed in protoscoleces 2 h after electroporation (d). No fluorescence was detected in the parasites of the untreated control (a) or parasites treated by soaking (b) 30 min after treatment. The scale bar indicates 200 μm.

The three promising candidate conditions, soaking with 10 µl transfection reagent, and electroporation with 100 V–800 µF and 200 V–100 µF, were tested for their efficacy of siRNA introduction using fluorescently labeled siRNA. For soaking, 100 µl culture medium containing approximately 2,000 protoscoleces was placed in a 24-well plate, and the fluorescently labeled siRNA (5 µM final concentration) alone or with 10 µl transfection reagent (NeoFX, Ambion) was then added to each well. The parasites were incubated at 37 °C in the presence of 5% CO_2 in the dark. For electroporation, 100 µl electroporation buffer (Ambion) containing approximately 2,000 protoscoleces was placed in a 4-mm cuvette, and the fluorescently labeled siRNA was added to give a final concentration of 5 µM. Electroporation was performed at 100 V–800 µF or 200 V–100 µF using an exponential decay pulse (Gene Pulser II, Bio-Rad). After incubation at 37 °C for 10 min, the buffer containing protoscoleces was transferred into 1 ml culture medium, which was then further incubated at 37 °C in 24-well plates in the presence of 5% CO_2 in the dark. At 30 min and 2 h after treatment, the parasites were washed with PBS and viewed under a fluorescent microscope (FV500, Olympus) to evaluate the efficacy of the three treatment conditions.

As expected, fluorescence was not detected in the untreated parasites (Fig. 1a). Surprisingly, fluorescence was also undetectable in the parasites treated by soaking with (Fig. 1b) or without transfection reagent (data not shown). We had previously hypothesized that the tegument, the surface syncytial structure that covers the entire body of the protoscolex and is involved in the active transport of extrasomatic low molecular weight nutrients [21], would be suitable for the absorption of siRNA. However, soaking proved to be unsuccessful for the introduction of siRNA into the parasites. In contrast, in the parasites treated by electroporation at 100 V–800 µF, strong fluorescence was detected 30 min after treatment (Fig. 1c), although the fluorescence decreased 2 h after treatment (Fig. 1d). Electroporation at 200 V–100 µF showed a very similar result to the 100 V–800 µF conditions (data not shown). The reason for the loss of fluorescence at 2 h after treatment is unknown. Krautz-Peterson et al. reported successful gene suppression in schistosomules using RNAi, although a similar reduction in fluorescence was observed in the method they used [9]. According to our present results, the electroporation condition of 100 V–800 µF was selected for the introduction of siRNA into protoscoleces in the subsequent experiments.

We next examined the efficacy of RNAi with the selected electroporation conditions. The respective target sequences were determined using the BLOCK-iT RNAi Designer software (https://rnaidesigner.invitrogen.com/rnaiexpress/), and the resulting 23 nt designed siRNA duplexes were obtained from Sigma-Aldrich at a concentration of 100 µM. The sequence of the siRNA targeting *14-3-3 zeta* was 5'-GCU CGU CGU UCA UCG UGG AGA AU-3' without a overhang, and that targeting *elp* was 5'-AAC CUU UCU AAG ACU GGA UAA GA-3' without a overhang. To analyze the effects of siRNA introduction into *E. multilocularis*, four sample groups of protoscoleces were prepared: untreated controls, electroporation controls, *14-3-3* siRNA-treated samples, and *elp* siRNA-treated samples. One hundred microliters of electroporation buffer (Ambion) containing approximately 2,000 protoscoleces was placed in a 4-mm cuvette for each sample. siRNA targeting *14-3-3* or *elp* at a final concentration of 3 µM was added to each cuvette of the 14-3-3 or *elp* siRNA-treated sample group, respectively. The negative control siRNA (Silencer negative control #1 siRNA, Ambion) was added to all of the electroporation controls at a final concentrations of 3 µM. Electroporation was then performed at 100 V–800 µF using an exponential decay pulse (Gene Pulser II, Bio-Rad). After incubation at 37 °C for 10 min, the buffer containing protoscoleces was transferred into 1 ml culture medium in the well of a 24-well plate. For the untreated controls, 2,000 protoscoleces were directly transferred from flasks into 1 ml culture medium. All samples were further incubated at 37 °C in the presence of 5% CO_2 in the dark and half of the medium was changed every 3 days. The analyses were performed in triplicate for each group.

The RNAi effects on mRNA levels were evaluated using real-time RT-PCR. For the analyses, total RNA was first extracted from all groups at day 0 and 3 after electroporation using TRIzol reagent (Invitrogen), and cDNA was then synthesized from 200 ng RNA in a total volume of 10 µl using the PrimeScript RT Reagent Kit (Takara) according to the manufacturer's instructions. Real-time PCR was carried out using 2 µl of 1:100 cDNA dilution, SYBR Premix Ex Taq II (Takara) and the following primer sets: *14-3-3 zeta* forward 5'-AAC TTG CTA TCC GTT GC-3', reverse 5'-CAC CTT CTT AAG GTA AAT GTC-3'; *elp* forward 5'-GTG AAG TCT GGT ACT TCG-3', reverse 5'-ATC CAG TCT TAG AAA GGT TG-3'; *actin 2* forward 5'-TCA ATC CTA AAG CCA ATC-3', reverse 5'-CGT ACA ACG ACA GCA C-3' for *emactin2* (XvEMb06047 from our cDNA library, http://fullmal.hgc.jp/em/); *14-3-3 epsilon* forward 5'-ATC TTA ATG ATG AAT CCG CTC CTG- 3', reverse 5'-GTT CAT CGC CCT CAT CCT TG- 3' for *14-3-3 epsilon* (Emmg-5f10.q1k, http://www.sanger.ac.uk/Projects/Echinococcus/). All samples were run in triplicate and underwent 40 amplification cycles at 95 °C for 5 s and 60 °C for 31 s using a StepOne Real-Time PCR System (Applied Biosystems). The amount of each cDNA was then calculated using standard curves and compared to the relative amounts of *emactin2*, an endogenous standard. Each relative amount was subsequently normalized to the untreated control at day 0. Statistical analyses using one-way ANOVA and Tukey's multiple comparison test were then performed.

The results of the real-time RT-PCR analyses revealed that *14-3-3 zeta* mRNA reduced to 21.8 ± 2.6% of the untreated control levels in *14-3-3* siRNA-treated protoscoleces by day 3, which was significantly ($P<0.01$) lower than the levels of the untreated control, electroporation control, and *elp* siRNA-treated samples (Fig. 2a). The levels of *elp* mRNA were also low in *elp* siRNA-treated protoscoleces, decreasing to 35.5 ± 0.4% of the untreated control levels by day 3, whereas the *elp* mRNA levels in the electroporation control and *14-3-3* siRNA-treated samples were 64.6 ± 19 and 57.5 ± 1.7% of the untreated control; however, ANOVA analyses suggested that the differences were not statistically significant (Fig. 2c). No significant reduction was observed in *14-3-3 epsilon* mRNA in any sample (Fig. 2b), even though *14-3-3 epsilon* mRNA contained a sequence which matches 21 of the 23 siRNA bases targeting the 14-3-3 zeta protein. This result suggested that as few as two base differences ensures the specificity of siRNA in *E. multilocularis*. All target mRNAs showed modest reductions in the electroporation controls; however, no statistical differences were observed between the untreated and electroporation controls.

The RNAi effects on protein expression levels were evaluated using western blot analysis. The treated protoscoleces were collected on day 3, 6, 10, and 15 after electroporation and homogenized using a hand homogenizer (23 M-R25, Nippon Genetics). Fifty microliters of radioimmunoprecipitation assay (RIPA) buffer containing 25 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS (pH 7.5) was added, and the specimens were treated by repeated freezing and thawing. Fifty microliters of 2× SDS sample buffer (100 mM Tris, 4% SDS, 12% 2-mercaptoethanol, 20% glycerol, and a few drops of bromphenol blue (BPB) solution (pH 6.8)) was added to the samples, which were then heated at 90 °C for 10 min and centrifuged at 20,000×g for 10 min. Extracted proteins were size separated on 12% acrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Detection of the specific proteins was performed using rabbit anti-14-3-3 [22], rabbit anti-antigen II/3 [20], and rabbit anti-actin (anti-beta-actin (NT), AnaSpec) antibodies as the primary antibodies, and goat anti-rabbit antibody (anti-rabbit IgG AP conjugate, Promega) as the secondary antibody. In the western blot obtained under reducing conditions, we identified a specific 27-kDa protein with the anti-14-3-3 antibody, 65-kDa and 52-kDa proteins with the anti-antigen II/3 antibody, and a 42-kDa protein with the anti-actin antibody. The detection of two II/3 protein bands has been reported previously by Felleisen & Gottstein, who speculated that

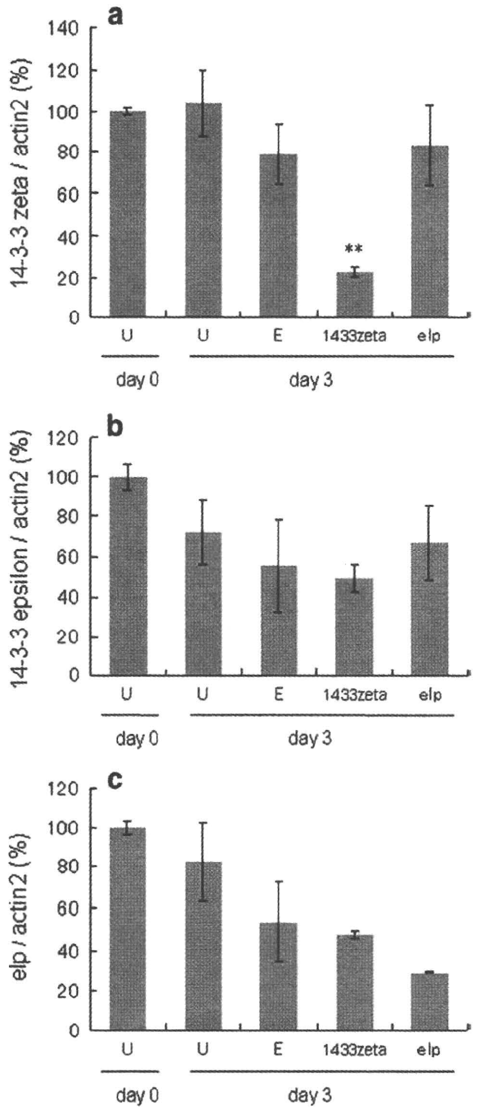

Fig. 2. RNAi effects on mRNA levels in *E. multilocularis* protoscoleces. Relative amounts of each mRNA were detected using real-time RT-PCR. *14-3-3 zeta* mRNA significantly reduced to 21.8 ± 2.6% of the untreated control levels in *14-3-3* siRNA-treated protoscoleces by day 3 (a). No significant reduction was observed in *14-3-3 epsilon* mRNA in any sample (b). The *elp* mRNA levels were lowered in *elp* siRNA-treated protoscoleces, reducing to 35.5 ± 0.4% of the untreated control by day 3. The electroporation control and *14-3-3* siRNA-treated samples showed *elp* mRNA levels of 64.6 ± 19 and 57.5 ± 1.7%, respectively, of the untreated controls. However, ANOVA analyses suggested that the differences between the siRNA-treated samples were not statistically significant (c). U: untreated control; E: electroporation control. Bars represent the standard deviation. $^{**}P<0.01$.

the 52-kDa protein could potentially be a processing or degradation product of the 65-kDa protein [19]. The PVDF membranes were air dried and scanned (Fig. 3a, b), and densitometry analyses were carried out using Photoshop 6.0 (Adobe). The values of the 14-3-3- and II/3-protein blots were normalized to those of actin and then transformed to set the untreated control of each day as 100%. The data were analyzed statistically using one-way ANOVA and Tukey's multiple comparison test. It was revealed that 14-3-3 protein gradually decreased to 41.6 ± 9.7, 39.7 ± 11.7, and 22.1 ± 7.1% of the untreated control levels by day 6, 10, and 15, respectively, in the *14-3-3* siRNA-treated samples. In the electroporation controls, 14-3-3 protein displayed a modest reduction compared to the untreated control from day 6 to 15 (70.0 ± 29.1 (day 6), 64.2 ± 15.0 (day 10), and 72.2 ± 5.2% (day 15) of the untreated control). Statistical analysis indicated that 14-3-3 protein was significantly reduced in siRNA-treated samples by day 15 (Fig. 3c). The densitometry analyses also demonstrated that the II/3-upper protein (65 kDa) levels were reduced to 88.2 ± 33.0, 87.2 ± 6.7, 59.6 ± 4.6, and 68.7 ± 4.6% of the untreated control by day 3, 6, 10, and 15, respectively, in the *elp* siRNA-treated samples. No reduction was observed in electroporation controls. Statistical analysis showed that II/3-upper protein was significantly reduced in the *elp* siRNA-treated samples by day 15 (Fig. 3d). The expression of the II/3-lower protein (52 kDa) was also decreased, with levels of 69.9 ± 5.5, 46.4 ± 5.2, 34.3 ± 2.7, and 26.2 ± 1.8% of untreated control observed by day 3, 6, 10, and 15, respectively, in the *elp* siRNA-treated samples. A modest effect was observed in the electroporation control by day 10 and 15, as the II/3-lower protein decreased to 78.5 ± 29.8 and 62.2 ± 6.0%, respectively, of the untreated control levels. Statistical analysis indicated that II/3-lower protein (52 kDa) was significantly reduced in the siRNA-treated samples on day 6 and 15 (Fig. 3e).

When a comparison was made with the electroporation controls that were set as 100%, the 14-3-3, II/3-upper (65 kDa), and II/3-lower (52 kDa) proteins reduced to 30.6 ± 9.9, 60.8 ± 4.0, and 42.1 ± 3.0%, respectively, in the corresponding siRNA-treated samples by day 15. It is speculated that the anti-14-3-3 antibody likely does not distinguish between the 14-3-3 isoforms, as it detected 14-3-3 isoforms other than what were targeted by the 14-3-3 zeta siRNA, indicating it is highly possible that 14-3-3 zeta itself was reduced to a greater extent than shown in these experiments. The results presented here suggest that RNAi did not uniformly affect the target genes in *E. multilocularis* protoscoleces. In this study, the knock-down of *14-3-3* was the most effective. The observed differences in the efficiency of RNAi on the *14-3-3* and *elp* (antigen II/3) mRNA and resulting protein levels may be due to differences of protein distribution within cells. It was previously reported that 14-3-3 protein mainly localizes in the apical region, pad and adjacent structures, and suckers of protoscoleces [15], whereas antigen II/3 localizes within the germinal layer and in the periphery of individual cell conglomerates inside of protoscoleces [19]. As shown in Fig. 1, it appeared that the amount of siRNA introduced into the surface structures was larger than that found in the internal regions of protoscoleces. It is therefore possible that more siRNA was delivered to cells actively producing 14-3-3 than to those cells producing antigen II/3. In addition to this speculation, the properties and stability of the target mRNAs and proteins may be involved in the difference knock-down efficiencies between the two proteins. The II/3-upper protein (65 kDa) seemed to be less sensitive to both electroporation and siRNA than the lower protein (52 kDa). Although the reason for the difference is unknown, again, the individual properties and stability of the two protein forms may be involved. Unexpectedly, we also observed protein reduction in a few of the electroporation control samples, suggesting that the electroporation treatment could affect the protein expression to a certain degree. It is considered that the reductions were a result of the electroporation procedure itself and were not caused by off-target effects of the negative control siRNA, since it was suggested that a mismatch of two nucleotides could ensure the specificity of siRNA (Fig. 2a and b), and a similar reduction was observed after electroporation without siRNA (data not shown). The differences of the electroporation treatment effects on the protein levels were likely due to differences in localization, stability, or other characteristics of these proteins. In future studies, it may be necessary to consider that observed reductions of target proteins in siRNA-treated samples would include effects by electroporation treatment, in addition to the RNAi effect, and appropriate controls should therefore be included.

Finally, the effects of siRNA introduction by electroporation on the viability of the protoscoleces were evaluated in all samples on day 3, 6, 10, and 15. Viability was calculated by counting the number of living protoscoleces that exhibited a clear appearance and that contained

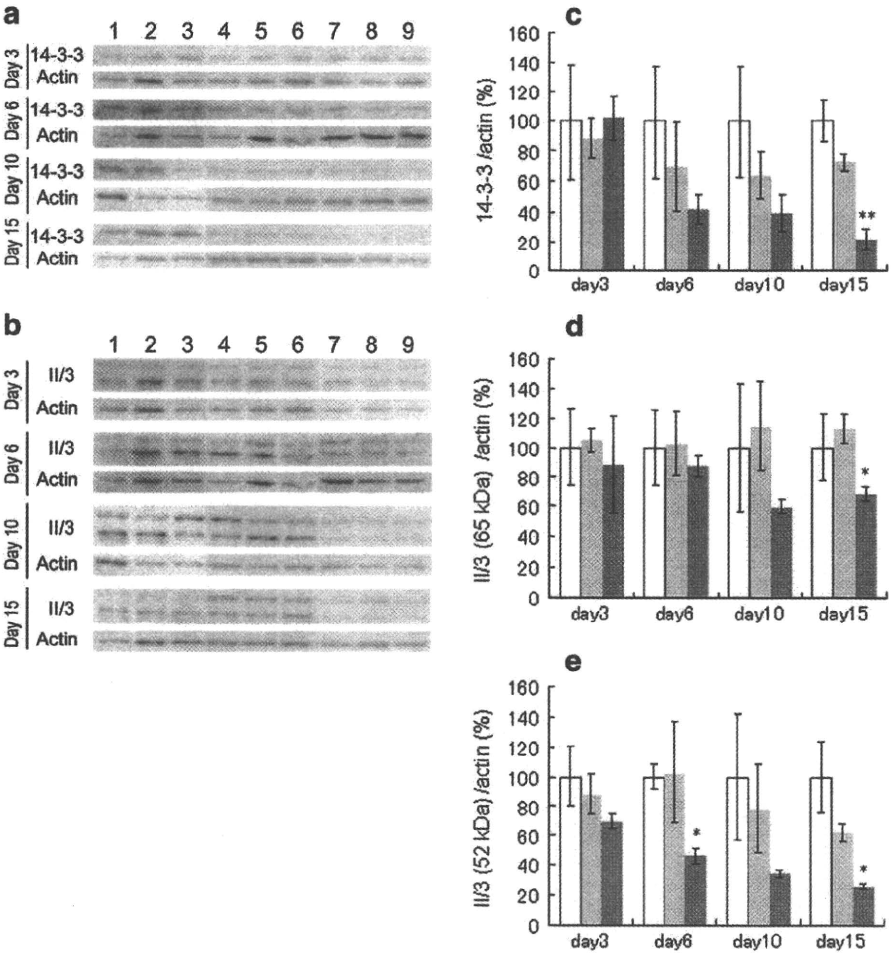

Fig. 3. RNAi effects on protein expression levels in *E. multilocularis* protoscoleces. Detection of 14-3-3, II/3 (*elp*), and actin was performed by western blotting. Untreated control (a and b, lanes 1–3), electroporation control (a and b, lanes 4–6), samples treated with siRNA targeting *14-3-3 zeta* (a, lanes 7–9), samples treated with siRNA targeting *elp* (b, lanes 7–9). The 14-3-3- and II/3-protein blots were evaluated by densitometry analyses. The values were normalized to those of actin and then transformed to set the untreated control levels of each day as 100%. The data were analyzed statistically by using one-way ANOVA and Tukey's multiple comparison test. 14-3-3 protein decreased to 41.6 ± 9.7, 39.7 ± 11.7, and 22.1 ± 7.1% of untreated control levels at day 6, 10, and 15, respectively, in the *14-3-3* siRNA-treated samples. (c). II/3-upper protein was reduced to 88.2 ± 33.0, 87.2 ± 6.7, 59.6 ± 4.6, and 68.7 ± 4.6% of the untreated control by day 3, 6, 10, and 15, respectively, in the *elp* siRNA-treated samples. No reduction was observed in the electroporation controls (d). II/3-lower protein decreased to 69.9 ± 5.5, 46.4 ± 5.2, 34.3 ± 2.7, and 26.2 ± 1.8% of the untreated control by day 3, 6, 10, and 15, respectively, in the *elp* siRNA-treated samples (e). $^{*}P<0.05$, $^{**}P<0.01$.

transparent structures and the number of dead protoscoleces that appeared opaque and demonstrated a rough surface and damaged inner structures. On day 3 and 6, no significant reduction of viability was observed in siRNA-treated samples. By day 10, the untreated control, electroporation control (silencer negative siRNA-treated), *14-3-3* siRNA-treated, and *elp* siRNA-treated samples displayed 94.6 ± 3.4, 86.5 ± 1.4, 80.1 ± 3.0, and 78.4 ± 4.0% viability, respectively, and by day 15, viabilities of 98.4 ± 1.4, 83.0 ± 2.5, 58.0 ± 23.0, and 55.1 ± 14.6%, respectively, were observed. The data of each day were analyzed by one-way ANOVA and Tukey's multiple comparison test, and a significant siRNA effect was found only between the controls (untreated and electroporation controls) and *elp* siRNA-treated samples on day 10. Despite the lack of statistical significance, the observed trend of decreasing viability in *14-3-3* and *elp* siRNA-treated protoscoleces indicates that these two proteins play important roles in essential cellular activities [14,18]. The 14-3-3 zeta protein is consider to have high potential as a vaccine candidate to larval echinococcosis [23], and a DNA vaccine consisting of *TEG-Tsag*, which is a homolog to *elp*, displayed significant levels of protection in a *Taenia crassiceps* murine model of cysticercosis [24].

In morphological observation, several protoscoleces increased in length and others swelled after the 15-day culture in both the control and treated groups. Although the number of swelled protoscoleces increased by a certain degree in samples treated by electroporation (controls and siRNA-treated), no clear difference was found by microscopic observation between the electroporation controls and siRNA-treated protoscoleces.

In conclusion, we demonstrated the potential of gene silencing in *E. multilocularis* protoscoleces using an RNAi method involving electroporation that induced reductions of target mRNA, target protein, and viability in *E. multilocularis* protoscoleces. Despite its successful application, further improvements to this method may be necessary, as it required relatively high concentrations of siRNA (3 or 5 μM), RNAi effects were not observed until several days after siRNA introduction, and the electroporation treatment may cause reductions in the levels of certain proteins. Nevertheless, this method represents a powerful tool for investigating gene function and identifying essential gene products in *E. multilocularis* protoscoleces.

Care of experimental animals

All animal experiments were carried out in accordance with the ethical guidelines of Hokkaido University and Hokkaido Prefecture.

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Development of PCR/dot blot assay for specific detection and differentiation of taeniid cestode eggs in canids

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ABSTRACT

We report the development of a colourimetric PCR/dot blot assay targeting the mitochondrial gene NADH dehydrogenase subunit 1 (*nad1*) for differential diagnosis of taeniid eggs. Partial sequences of the cestode *nad1* gene were aligned and new primers were designed based on conserved regions. Species-specific oligonucleotide probes (S-SONP) for canine taeniid cestodes were then designed manually based on the variable region between the conserved primers. Specifically, S-SONP were designed for the *Taenia crassiceps*, *T. hydatigena*, *T. multiceps*, *T. ovis*, *T. taeniaeformis*, *Echinococcus granulosus* (genotype 1), *E. multilocularis* and *E. vogeli*. Each probe showed high specificity as no cross-hybridisation with any amplified *nad1* fragment was observed. We evaluated the assay using 49 taeniid egg-positive samples collected from dogs in Zambia. DNA from 5 to 10 eggs was extracted in each sample. Using the PCR/dot blot assay, the probes successfully detected PCR products from *T. hydatigena* in 42 samples, *T. multiceps* in 3 samples, and both species (mixed infection) in the remaining 4 samples. The results indicate that the PCR/dot blot assay is a reliable alternative for differential diagnosis of taeniid eggs in faecal samples.

1. Introduction

Canids, such as dogs, dingoes, foxes, wolves and jackals, harbour the adult stage of important taeniid cestode species, including *Echinococcus granulosus*, *E. multilocularis*, *Taenia ovis*, *T. multiceps* and *T. hydatigena*. Whereas the dangers caused by larval stages of *Echinococcus* species to public health are well known [1], the potential risk associated with zoonotic infection by metacestodes of several *Taenia* spp. such as *T. multiuceps* (*Coenurus cerebralis*) [2] and *T. crassiceps* (*Cysticecus longicollis*) [3] is poorly clarified. In fact, several *Taenia* species have been reported to be highly prevalent in many countries including Uruguay [4], Ethiopia [5], and Italy [6] where tons of carcasses and offal are discarded every year due to infection of domestic livestock by taeniid larvae.

The lack of accurate diagnostic methods for *Taenia* species differentiation in live canids further hinders our understanding of the biology and host-parasite interaction of these parasites. Since canids can harbour several species of *Taenia* and *Echinococcus* simultaneously, developing a method for detecting and distinguishing between taeniid eggs present in faeces is considered to be essential. While the detection of coproantigens by ELISA (coproELISA) [7,8] and PCR (copro-DNA) [9] had previously been used to accurately diagnose infection by *Echinococcus* spp., analogous methods for distinguishing between species of *Taenia* in canids have not yet been developed. Although Gasser and Chilton [10] and Trachsel et al. [11] successfully discriminated *Taenia* spp. by PCR-RFLP, their methods had not been used in survey studies.

The PCR/dot blot assay is a widely used hybridisation technique that has been applied to the identification and genotyping of pathogens such as *Mycobacterium tuberculosis* [12], *Chlamydia psittaci* [13], and Echovirus [14]. The simplicity of this hybridisation assay enables simultaneous and rapid screening of several samples and is capable of species differentiation using species-specific oligonucleotide probes. Lavikainen et al. [15] reported that *Taenia* spp. can be differentiated based on the polymorphisms of the cytochrome *c* oxidase subunit 1 (*cox1*) gene and NADH dehydrogenase subunit 1 (*nad1*) gene sequences. Among these two genes, we chose *nad1* as a candidate for oligonucleotide probes design on the basis of the higher variability range [16]. In addition much more sequences of this gene are currently registered in the GenBank database compared to other genes studied to date.

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The aim of this study was therefore to develop a colourimetric PCR/dot blot assay using species-specific oligonucleotide probes targeting the *nad1* gene to detect and identify taeniid eggs present in canid faeces.

2. Materials and methods

2.1. Parasite sample collection

Cestode adults and larvae used in this study were collected from a variety of hosts in several countries (Table 1). After collection and identification using morphological characters under a light microscope, samples were stored in 70% ethanol until use. Taeniid eggs were isolated from 49 dog faeces samples collected in Lusaka and the Eastern province of Zambia from 2005 to 2007. Briefly, the faecal samples were collected from the rectums of each dog and stored at −80 °C for 10 days and then at −40 °C until egg isolation. Then, 0.5 g of faeces from each dog was placed into a 15 ml plastic tube (Asahi Glass Co. Ltd., Japan) and suspended in sucrose solution (specific gravity: 1.27). After 10 min of centrifugation at 2000 rpm the tubes were laid vertically and filled to the edge of the bottle opening with additional sucrose solution. A glass coverslip was then placed on the top of each tube and left for 1 h to allow the eggs to float to the surface and attach to it. Five to ten taeniid eggs were then manually recovered from the glass coverslips under a stereomicroscope, placed in double distilled water (DDW), and stored at −40 °C until use.

2.2. DNA extraction

Genomic DNA was extracted from adult cestodes, larvae and eggs using QIAamp DNA mini kit (Qiagen K.K., Japan) following the manufacturer's instructions. The concentration of extracted DNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., USA).

2.3. PCR amplification of nad1 gene

Common primers for the taeniid cestode *nad1* gene, *nad1*T-Fw (5′-GGK TAT TCT CAR TTT CGT AAG GG-3′) and *nad1*T-Rv (5′-ATC AAA TGG AGT ACG ATT AGT YTC AC-3′), were designed based on the conserved regions of 11 *Echinococcus* taxa and 10 *Taenia* taxa registered in GenBank (Table 2). The expected size of the amplicons was 507 bp. PCR amplifications were conducted in a reaction mixture consisting of 5 µl of 10× Ex *Taq* buffer, 2.5 mM of each dNTP, 1 U of Ex Taq™ (Takara Bio Inc., Japan), 1 µM of each primer (Hokkaido System Science Co. Ltd., Japan), 5 µl template DNA and up to 50 µl DDW. Amplification was performed using a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA) programmed for 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 1 min. Five microlitres of PCR products were fractionated by electrophoresis on a 1.5% agarose gel in TAE buffer (40 mM Tris, 40 mM acetic acid, and 1 mM EDTA). The gels were then stained in a 1 µg/ml ethidium bromide solution before being photographed under UV light. The remaining 45 µl of PCR product were purified using QIAquick PCR purification kit (Qiagen K.K., Japan) following the manufacturer's instructions and stored at −40 °C until use. The concentration of the PCR products was measured using a NanoDrop 1000 spectrophotometer.

Table 1
Host and country of origin of the cestode species (adult/larvae) used in this study.

Species	Abbreviation	Country	Host
Dipylidium caninum	Dc	Uruguay	Dog
Mesocestoides vogae	Mv	Switzerland	Vole
Echinococcus granulosus (*genotype 1*)	Eg#1	Mauritania	Camel
Echinococcus granulosus (*genotype 1*)	Eg#2	Uruguay	Dog
Echinococcus canadensis (*genotype 6*)	Ec	Zambia	Cattle
Echinococcus multilocularis	Em#1	Austria	Vole
Echinococcus multilocularis	Em#2	Japan	Vole
Echinococcus multilocularis	Em#3	France	Human
Echinococcus multilocularis	Em#4	Japan	Vole
Echinococcus vogeli	Ev	Colombia	*Agouti paca*
Taenia hydatigena	Th#1	Japan	Dog
Taenia hydatigena	Th#2	China	Dog
Taenia hydatigena	Th#3	Japan	Sheep
Taenia hydatigena	Th#4	Switzerland	Dog
Taenia hydatigena	Th#5	Uruguay	Dog
Taenia crassiceps	Tc#1	Japan	Fox
Taenia crassiceps	Tc#2	Japan	Vole
Taenia multiceps	Tm#1	China	Dog
Taenia multiceps	Tm#2	China	Sheep
Taenia ovis	To	Switzerland	Dog
Taenia taeniaeformis	Tt#1	France	Vole
Taenia taeniaeformis	Tt#2	France	Vole
Taenia taeniaeformis	Tt#3	Japan	Rat

Table 2
Accession numbers of taeniid cestode nad1 gene sequences used for designing primers and oligonucleotide probes.

Parasite	Accession number
Echinococcus granulosus (*genotype 1*)	AF297617
Echinococcus equinus (*genotype 4*)	AF346403
Echinococcus ortleppi (*genotype 5*)	AB235846
Echinococcus canadensis (*genotype 6*)	AB208063
Echinococcus canadensis (*genotype 8*)	AB235848
Echinococcus canadensis (*genotype 9*)	AB235847
Echinococcus canadensis (*genotype 10*)	AF525297
Echinococcus multilocularis	AB018440
Echinococcus oligarthrus	AB208545
Echinococcus shiquicus	AB208064
Echinococcus vogeli	AB208546
Taenia crassiceps	AF216699
Taenia hydatigena	DQ995654
Taenia multiceps	AY669089
Taenia ovis	AJ239103
Taenia pisiformis	AJ239109
Taenia polyacantha	DQ408420
Taenia saginata	AY684274
Taenia serialis	DQ401137
Taenia solium	AB086256
Taenia taeniaeformis	EF179171

2.4. DNA sequencing and homology search

The sequences of PCR products were determined by direct sequencing using an automated sequencer (CEQ 8000, Beckman Coulter Inc., USA) and a GenomeLab™ DTCS quick start kit for dye terminator cycle sequencing (Beckman Coulter Inc., USA). A homology search of the obtained sequences was performed by conducting an online NCBI Basic Local Alignment Search Tool for nucleotides (BLASTN) search of the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Oligonucleotide probes

The species-specific oligonucleotide probes were designed by eye based on *nad1* sequences retrieved from the GenBank database. The theoretical specificity of the probes was assessed by a sequence homology search with the BLASTN algorithm. The probes designed in this study were labeled with digoxigenin (DIG) using the DIG oligonucleotide 3′-end labeling kit, 2nd Generation (Roche Diagnostics, Germany) and stored at −40 °C until use.

Table 3
List of species-specific oligonucleotide probes designed in this study.

Parasite	Probe name	Sequence (5′-3′)
E. granulosus (genotype 1)	EgG1-*nad1*	CCGCCAGAACATCTAGGTATT
E. multilocularis	Em-*nad1*	TTTGTTCTTTGTGTTACTGTAGGTA
E. vogeli	Ev-*nad1*	TGTTATGATTCTTAGCTGCTGC
T. crassiceps	Tcra-*nad1*	GTACGTAGAAATTATAGTTTATTAGGAGC
T. hydatigena	Thyd-*nad1*	GTTTATGGGTCTTATCATAGTTGTAG
T. multiceps	Tmul-*nad1*	TGTATATTATTCTTTTGTATATGGTGGTT
T. ovis	Tov-*nad1*	TGGTGTGATATTACTTGTTAATTTAGTT
T. taeniaeformis	Ttae-*nad1*	TTTATGTGGTTATGCTGTGTTATGT

2.6. Dot blot assay

Dot blot assays were carried out using the DIG nucleotide acid detection kit (Roche Diagnostics). Briefly, the *nad1* PCR products were heated at 95 °C for 10 min before being cooled on ice. Two microlitres of each denatured PCR product was then manually blotted in duplicate on positively charged nylon membranes previously activated with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) for 5 min. The membranes were then soaked in 0.4 M NaOH for 5 min, rinsed with 2× SSC for 10 min, and dried before use. The blots were hybridised with 10 pM oligonucleotide probes at 60 °C for 1.5 h. After hybridisation, each blot was washed twice for 5 min in 2× SSC/0.1% SDS (sodium dodecyl sulphate) at room temperature (RT) with gentle agitation, followed by additional two washes with pre-heated 0.5× SSC/0.1% SDS for 10 min at 60 °C. Blots were then washed in DIG Wash and Block Buffer Set for 2 min at RT before the blocking reaction was performed by incubating in blocking solution for 30 min. After the membranes were incubated in antibody solution containing 150 mU/ml alkaline phosphatase conjugated anti-digoxigenin antibody (anti-digoxigenin-AP) for another 30 min, they were washed twice in washing solution for 15 min before being equilibrated in 0.1 M Tris–HCl and 0.1 M NaCl (pH 9.5) for 3 min. For colourimetric detection of hybridisation, substrate solution (NBT/BCIP) was used as per the manufacturer's instructions. Colour development was performed for 1 h and the reaction stopped by washing the membrane with TE buffer (10 mM Tris–HCl (pH 8.0), and 1 mM EDTA) for 5 min.

3. Results

3.1. nad1 PCR with newly designed primers

The specificity of the new primer pair, *nad1*T-Fw/*nad1*T-Rv, was evaluated by using adult/larval cestode genomic DNAs as templates (Table 1). Fragments of the *nad1* gene were amplified from all of the taeniid cestode DNAs tested, as well as two non-taeniid cestodes, *Dipylidium caninum* and *Mesocestoides vogae*. Sensitivity tests of the

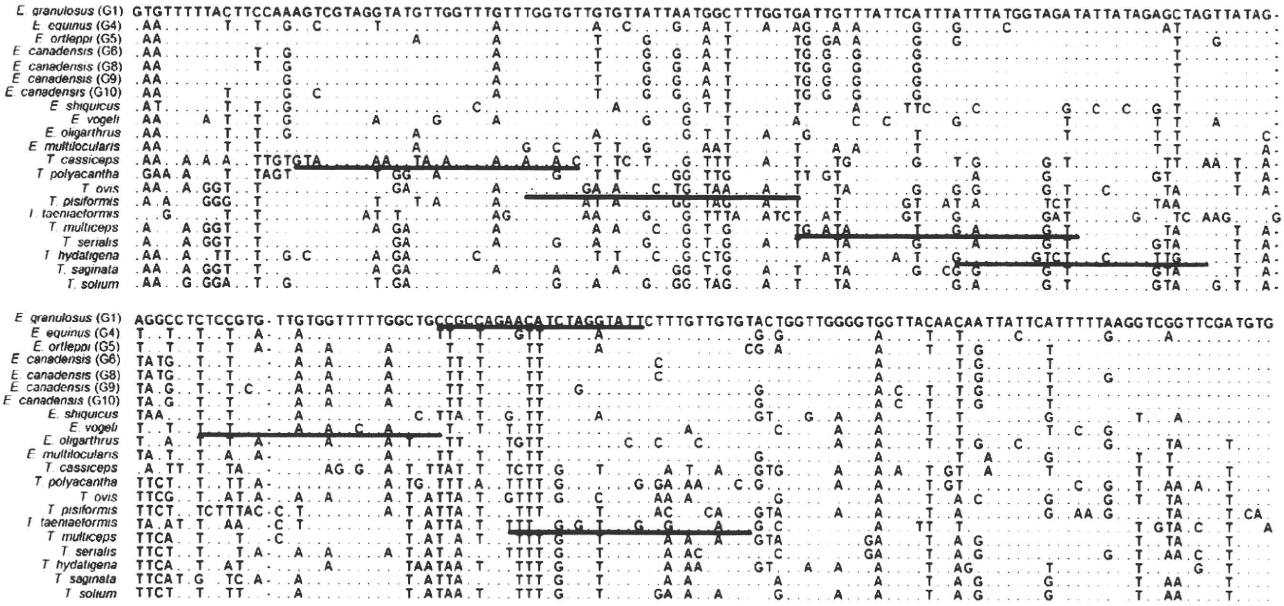

Fig. 1. Multiple alignment of the partial *nad1* sequences containing variable regions in different taeniid species. Sequences were obtained from GenBank. The position of species-specific oligonucleotide probes were underlined and dots represent bases identical to those in the *E. granulosus* genotype 1 sequence (first line).

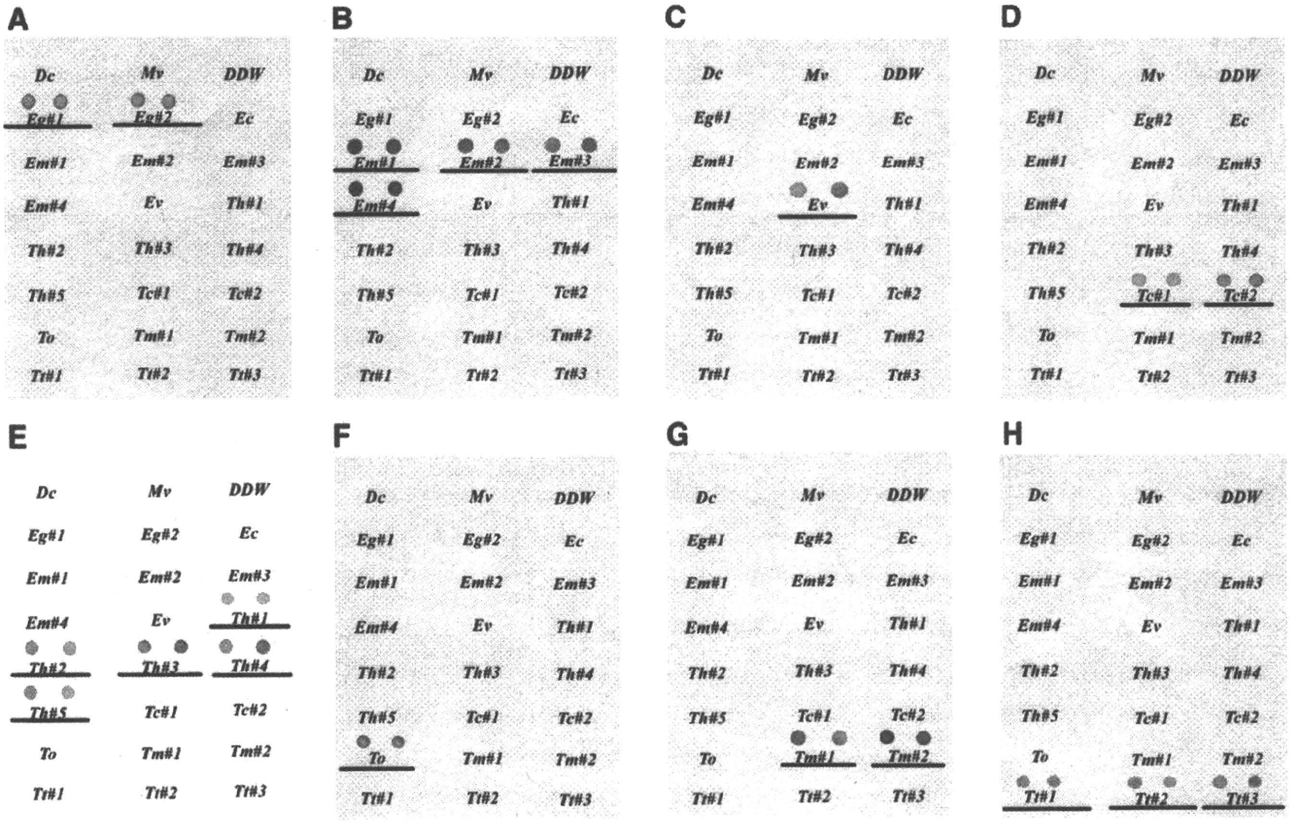

Fig. 2. Dot blot assay performed using adult/larval cestode *nad1* PCR products. Membranes A to H correspond to the *E. granulosus* (genotype 1), *E. multilocularis*, *E. vogeli*, *T. crassiceps*, *T. hydatigena*, *T. ovis*, *T. multiceps* and *T. taeniaeformis* probes, respectively. The PCR products blotted on these membranes are (from top to bottom) Dc, *D. caninum*; Mv, *M. vogae*; Eg, *E. granulosus* (genotype 1); Ec, *E. canadensis* (genotype 6); Em, *E. multilocularis*; Ev, *E. vogeli*; Tc, *T. crassiceps*; Th, *T. hydatigena*; To, *T. ovis*; Tm, *T. multiceps* and Tt, *T. taeniaeformis*. The species with more than one sample were numbered. Underline indicates the species-specific hybridisation. *D. caninum* and *M. vogae nad1* PCR products were included as negative control DNAs and DDW was applied in the blank space. No cross-reactions were observed between any of the probes.

primer pair was performed using serial dilutions of the cestode genomic DNA templates, revealing that 5 pg was sufficient for amplifying the *nad1* fragment in all of the species assayed (data not shown).

3.2. Oligonucleotide probes

DNA sequences of *nad1* genes registered in the GenBank database (Table 2) were aligned using BioEdit version 7.0.0. The oligonucleotide

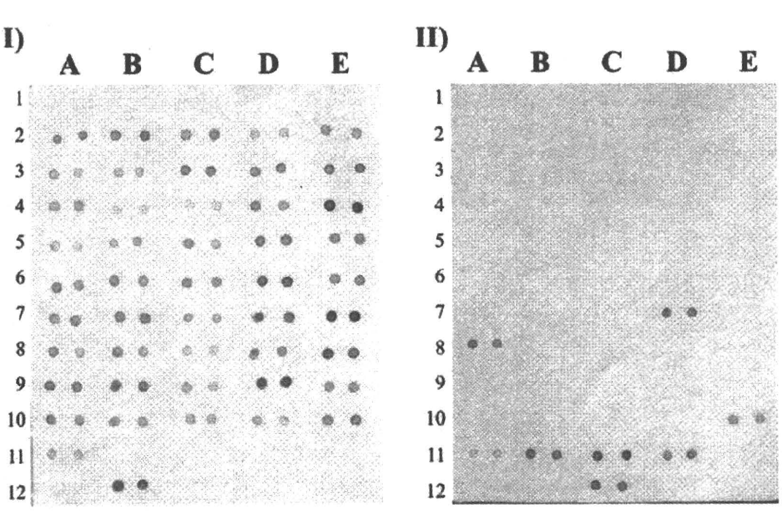

Fig. 3. Dot blot assay of 49 taeniid egg samples. Membrane I) was hybridised with *T. hydatigena* probe, and II) was hybridised with *T. multiceps* probe. As controls, PCR products of *E. granulosus* genotype 1, *E. canadensis* genotype 6, *E. multilocularis* and *E. vogeli* were applied to the first row (1A–1D), and PCR products from *T. crassiceps*, *T. hydatigena*, *T. multiceps*, *T. ovis* and *T. taeniaeformis* were applied to the bottom row (12A–12E). Samples were organised based on PCR/direct sequencing results with *T. hydatigena* samples on row 2A–10E and *T. multiceps* on row 11A–11D. DDW was applied on 1E and 11E. Of the 49 samples, 42 hybridised with *T. hydatigena* probe and 3 with *T. multiceps* probe, the other 4 samples (7D, 8A, 10E, and 11A) reacted with both *T. hydatigena* and *T. multiceps* probes.

probe candidates (Table 3) were selected based on whether they exhibited similar properties (i.e. predicted melting temperature and length), but showed the greatest differences in sequence among the species assayed (Fig. 1).

3.3. Evaluation of probes

As shown in Fig. 2, the specificity of each probe was tested using the *nad1* fragments amplified from adult/larval DNA. The results showed that probes specifically hybridised with the *nad1* fragments amplified using the newly designed primers, with no cross hybridisation observed. To evaluate the detection limit, the PCR products were serially diluted up to 1 ng/µl and subjected to the PCR/dot blot assay. The resulting blots revealed that 2 ng of blotted PCR product could reliably be detected (data not shown).

3.4. Egg differentiation

The *nad1* gene fragment of the 49 egg-positive samples was PCR-amplified and subjected to dot blot analysis using all of the probes. Of the 49 samples tested, 42 *nad1* gene fragments hybridised with the *T. hydatigena* probe, 3 with the *T. multiceps* probe and the remaining 4 with both *T. hydatigena* and *T. multiceps* probes (Fig. 3). The other probes did not hybridise with any of the samples.

4. Discussion

Although the PCR-RFLP assays have been shown to be highly sensitive [10,11], they have limitations in terms of time and labour, combined with the restrictions on the number of samples that can be processed simultaneously, complicating the widespread adoption of this technique using field samples.

Mitochondrial DNA sequencing has been used extensively to identify a variety of organisms, including parasites, and the *cox1* and *nad1* genes have frequently been used in phylogenetic studies [15,17,18]. In the case of taeniid species, the *cox1* gene had been shown to be more conserved than the *nad1* gene, with inter-taxon differences in both genes observed to range from 2.5 to 18% and 5.9 to 30.8%, respectively [16]. We therefore targeted the *nad1* gene for amplification and subsequent analysis using the dot blot detection assay with species-specific oligonucleotide probes to distinguish between taeniid species.

Most of taeniid *nad1* sequences registered in Genbank had been generated using the primer set JB11/JB12 [19]. However, we could not amplify several taeniid samples using this primer pair (data not shown). Therefore, a new set of PCR primers for the cestode *nad1* gene (*nad1*T-Fw and *nad1*T-Rv) was designed next to JB11/JB12 to facilitate the alignment and comparison with published sequences. The PCR amplification using the *nad1*T-Fw/*nad1*T-Rv primers yielded products of expected size. In addition, specific amplification of each *nad1* gene fragment was confirmed by sequence analysis followed by a homology search (data not shown).

Based on nucleotide differences in the *nad1* gene, several oligonucleotide probe candidates were designed within the variable region flank by the newly designed primers. When the oligonucleotide probes were evaluated by the PCR/dot blot assay using *nad1* PCR fragments from adult/larval DNAs, the high specificity of eight probes (Table 3) was clearly demonstrated and no cross hybridisation was observed. The detection limit of the *nad1* PCR products for each probe was 2 ng, indicating that the assay was highly sensitive.

Finally, we evaluated the PCR/dot blot assay using *nad1* fragments PCR-amplified from egg DNAs. In the PCR/dot blot system developed in this study, 42 samples hybridised only with *T. hydatigena* probe and 3 with *T. multiceps* probe, while the remaining 4 samples hybridised with both *T. hydatigena* and *T. multiceps* probes. We conclude that these 4 samples harboured eggs of both *T. hydatigena* and *T. multiceps*. Sensitivity tests of the PCR assay described herein revealed that the minimum amount of DNA template required for *nad1* gene amplification was 5 pg (data not shown). Since the amount of DNA in a single egg was reported to be 8 pg [20], the sensitivity of our PCR assay is considered to be sufficiently high to amplify the *nad1* gene from a single egg. Moreover, the PCR/dot blot assay was able to detect and differentiate between DNA extracted from five eggs. These findings suggest that the PCR/dot blot assay has the following advantages; 1) *easy-to-perform*; after PCR, no special equipment is required and results can be assessed by the naked eye, 2) *detectability of mixed infection*; simultaneous infection by several taeniid cestodes can be detected using species-specific probes, and additionally 3) *cost-effectivity*; reuse of hybridisation buffers containing probes could markedly reduce the cost of performing the assay.

In conclusion, the PCR/dot blot assay presented herein enables the detection and differentiation of eight of the most important taeniid cestodes in the areas of veterinary and public health. Moreover, this technique can be performed with relative ease in laboratories with thermal cyclers. We expect that PCR/dot blot assay described in the present study would be an alternative method for taeniid egg differentiation collected in the field. In order to improve the method, especially for eliminating egg purification step, further studies have to be conducted using coproDNA.

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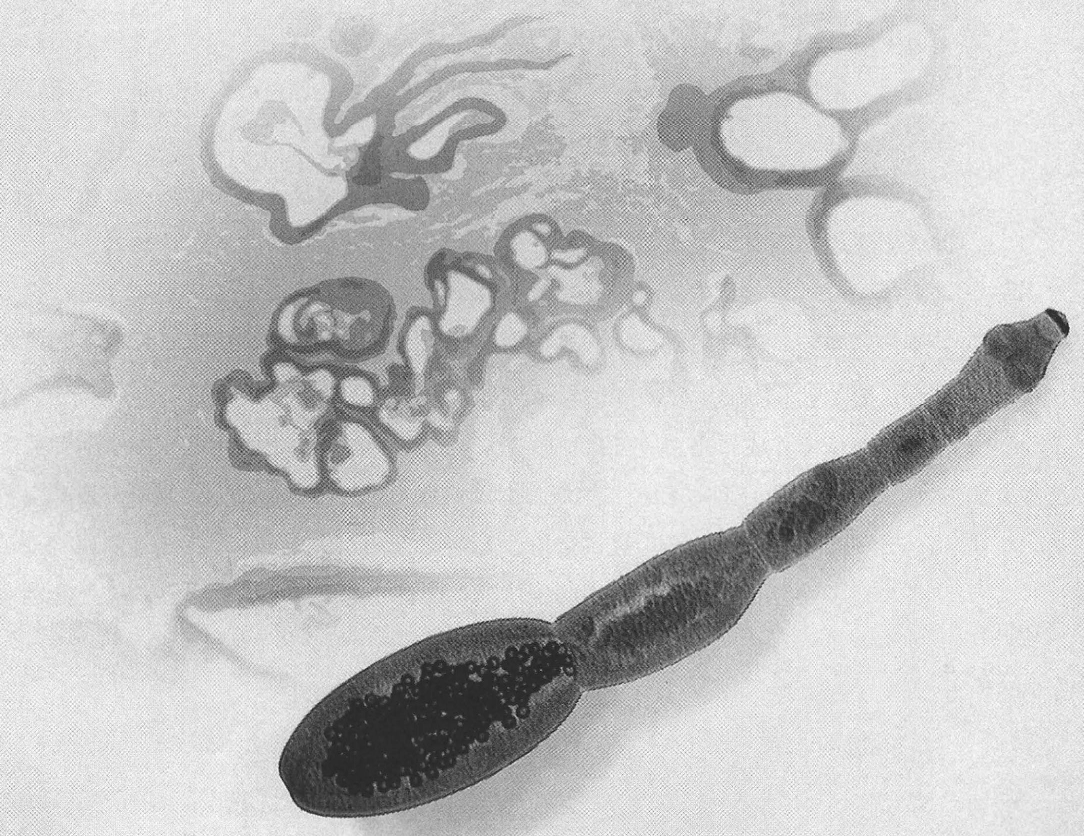
特別振興事業

人獣共通寄生虫の
エキノコックス症

Echinococcosis



社団法人 中央畜産会

発刊にあたって

エキノコックス症は、多包条虫 *Echinococcus multilocularis* が起こす寄生虫病で、主にキツネと野ネズミ間で伝播している野生動物の寄生虫であるが、犬、豚、牛及び人へも感染する人獣共通寄生虫であり、わが国においては、北海道及び東北地域の一部で、発生が知られている。また、多包条虫に近縁な単包条虫*Echinococcus granulosus* は全世界的に分布し、野ネズミではなく家畜および野生の有蹄獣に感染し、特に畜産国で問題となっている。世界の患者数については、単包条虫は200～300万人、多包条虫は30万人程度と見積もられ、世界的には単包条虫がより重要であり、日本国内でも時折輸入家畜から発見されている。人のエキノコックス症では多包条虫が、肝臓でゆっくりと無性増殖し、重篤な慢性疾患を引き起こし、適切な治療を施さない場合、高い致死率を示すといわれている。北海道のキツネの感染率は全道平均で40%にも達し、飼犬からも寄生例が時折見つかり、今後の北海道における患者数の増加と、本州への流行地拡大も危惧されているため、その発生予防に留意すべき伝染病となっております。

この冊子は、日本中央競馬会の振興基金による財団法人全国競馬・畜産振興会の助成事業の平成21年度家畜衛生体制強化推進事業（馬インフルエンザ等自衛防疫推進事業）の一環として、獣医師等関係者が、今後とも留意していかなければならない本病についての病因、疫学、対策等を総括的にとりまとめ、本病に係る知識の普及を目的として作成したものです。

この冊子の作成に当たっては、国立大学法人北海道大学大学院獣医学研究科寄生虫学教室の奥 祐三郎准教授に執筆をお願い致しました。また、貴重な写真については、奥 祐三郎准教授からご提供を頂きました。

この冊子が今後の家畜伝染病防疫体制を構築する上での一助となることを願っております。

平成22年2月

社団法人 中央畜産会会長
小里　貞利

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人獣共通寄生虫のエキノコックス症

はじめに

寄生虫の多くは宿主に対し病原性をあまり示さないが、まれに重篤な疾病を引き起こす場合がある。北海道で問題となっている多包条虫 *Echinococcus multilocularis* は主にキツネと野ネズミ間で伝播している野生動物の寄生虫であるが、人へも感染する人獣共通寄生虫であり、患者では適切な治療を施さない場合高い致死率を示す。人は多包条虫にとって好適な宿主ではないが、肝臓でゆっくりと無性増殖し、重篤な慢性疾患を引き起こす。1930年代以降本寄生虫が流行した礼文島（当時の島民約1万）では約130名の患者が発見されたが、2000年以降の北海道（人口約557万人）における年間患者発生数は平均18名である。しかし、北海道のキツネの感染率は全道平均で40%にも達し、飼犬からも寄生例が時折見つかり、今後の北海道における患者数の増加と、本州への流行地拡大も危惧されている。

多包条虫に近縁な単包条虫 *Echinococcus granulosus* は全世界的に分布し、野ネズミではなく家畜および野生の有蹄獣に感染し、特に畜産国で問題となっている。世界の患者数については、単包条虫は200～300万人、多包条虫は30万人程度と見積もられ、世界的には単包条虫がより重要であり、日本国内でも時折輸入家畜から発見される。本冊子ではこれらの単包条虫と多包条虫を含むエキノコックスについて述べる。

エキノコックスとは

寄生虫学では、寄生虫が性成熟に達し、有性生殖を行うまで発育できる宿主を終宿主、幼虫の発育に必要な宿主を中間宿主と呼んでいる。エキノコックス *Echinococcus* は扁形動物門、条虫綱、円葉条虫目、テニア科に属する寄生虫である。一般に条虫類は脊椎動物を終宿主とし、無脊椎動物を中間宿主として利用するが、テニア科条虫は肉食動物を終宿主とし、それらに捕食されるほ乳類を中間宿主とする。さらに、エキノコックスは中間宿主体内で無性増殖し、条虫の中でも特異な条虫である。成虫は細長く、3-6節の片節を有し、大きさは、体長7mm以下の小型の寄生虫である（図1）。頭部は頭節があり、ここには多数の鉤と4つの吸盤があり、腸粘膜に吸着している。一方、幼虫は肺や肝臓実質に寄生し、袋状で大きく、包虫（hydatid）と呼ばれる。包虫は単純な胞状の単包型（unilocular）から、やや小さな袋が集まった多胞型（polycystic）、さらに微細な嚢胞の集塊の多包型（alveolar, multivesicular）など

図1. エキノコックス（多包条虫）の成虫と虫卵