

reference positions of lipoproteins were identified by staining with Coomassie Brilliant Blue and prestaining with Sudan black.

RESULTS

Dependence on HDL of *S. japonicum* egg embryonation

Maturation and embryonation of *S. japonicum* eggs was investigated during the culture by counting formation of miracidia. The eggs were cultured with or without 10% pooled human serum for 8 d after separation from the parent adults precultured for 2 d in medium containing 5 or 10% serum. Embryonation significantly decreased without serum supplementation when the eggs were laid by parents precultured in 5% serum, while the maturation proceeded even without serum when the parent flukes were precultured in 10% serum (15.8 vs. 38.0%; Fig. 1A). When the apoB-LP-deficient serum (bottom fraction of $d=1.063$ g/ml) was used for culture of the eggs, the eggs from the 5% serum-treated parents achieved the similar level of embryonation (Fig. 1B). Egg embryonation was estimated in the medium after pairs of *S. japonicum* worms were incubated for 10 d with and without HDL fraction ($1.063 < d < 1.21$) in addition to the lipoprotein-deficient serum ($d > 1.21$) at the equivalent concentration of 10% serum. Egg embryonation by HDL reached a similar level to that by 10% serum, but lipoprotein deficient serum alone showed little or no maturation (Fig. 1C).

The results indicated that the egg maturation requires serum nutrients. Preconditioning of the parents or the eggs in the early stage by exposure to well-supplemented nourishment overcomes insufficient nourishment in the later stage. Nutritional support for the adults is perhaps primarily essential to deposit egg yolk/vitelline materials for embryonation, but the eggs also are able to take up nutrients from serum to grow even when they were laid in an insufficiently nourished condition. ApoB-LPs were shown not to be functional, but a normal HDL fraction is essential for egg maturation. Interestingly, minimal embryonation of the eggs was seen in culture medium containing 10% serum from wild-type mice, which is deficient in CETP,

whereas the maturation proceeded well in medium containing serum from CETP-transgenic mice or wild-type mouse serum presupplemented with human CETP (ref. 24 and Fig. 1D), supporting our previous findings (10).

Uptake of cholesterol and cholesteryl ester from HDL *S. japonicum* eggs

To characterize association of HDL with the *S. japonicum* eggs, HDL was labeled with [³H]cholesteryl ester or ¹²⁵I on the HDL protein, and association of their radioactivity with *S. japonicum* eggs was determined. Apparent association of HDL was estimated as its protein based on specific radioactivity of HDL. As shown in Fig. 2A, [³H]-based association, assessed as increment from 4 to 37°C, was markedly higher than that of ¹²⁵I binding, which showed no difference between 37 and 4°C, indicating that no active HDL protein processing was involved, suggesting that cholesteryl ester is selectively taken up by the eggs. As the concentration of HDL increased, the ¹²⁵I binding seemed saturated at lower concentrations of HDL (100 µg/ml) than the [³H]-based association ($>>600$ µg/ml). The egg takes up cholesteryl ester also from apoB-LP, but the rate seems saturated at lower concentrations (150 µg/ml) with less maximum uptake than HDL, based on cholesteryl ester (Fig. 2A, right panel). Uptake by schistosome eggs of free and esterified cholesterol was examined by using double-labeled HDL and apoB-LP with [¹⁴C]cholesterol and [³H]cholesteryl ester. Uptake of [¹⁴C]cholesterol was impeded by excess apoB-LP and HDL, but the uptake of [³H]cholesteryl ester was suppressed only by HDL and not by apoB-LP (Fig. 2B). This profile indicated a specific pathway for selective uptake of cholesteryl ester from HDL, at least different from a pathway for the uptake of apoB-LP cholesteryl ester (25), while free cholesterol uptake is nonspecific from LDL and HDL, including its exchange among lipoproteins and cell membranes.

Northern blot analysis

Assuming that selective uptake is mediated by SR-BI-like protein or CD36 family protein, expression of

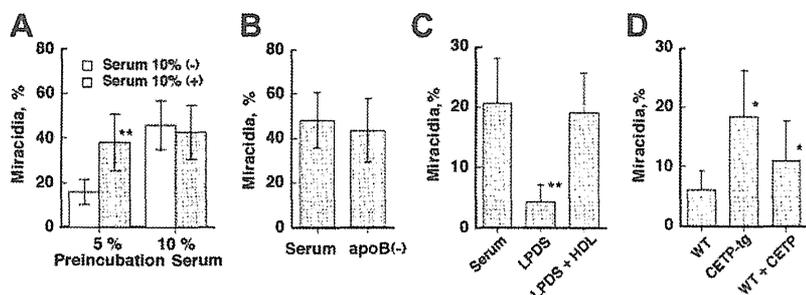


Figure 1. Embryonation and maturation of the *S. japonicum* eggs in culture. The percentage of miracidia in total eggs per pair of adult parasites cultured for 10 d was counted and estimated as efficiency of embryonation. A) Eggs were separated from the adult worms after 2 d of culture in RPMI 1640 medium supplemented with 5 or 10% human pooled serum, and cultured further in fresh medium supplemented with or without 10% serum for 8 d. B) Eggs were separated from the parents cultured in the same condition as in A with 5% serum, and the eggs were further cultured in 5% CO₂ atmosphere with 10% of whole serum and its $d = 1.063$ bottom fraction (apoB(-)). C) A pair of parent adults was cultured for 10 d in the medium with lipoprotein-depleted serum (LPDS; 4 mg protein/ml) with or without isolated HDL fraction (150 µg cholesterol/ml). D) A pair of *S. japonicum* adults was cultured with mouse sera (4 mg protein/ml) of wild-type, CETP transgenic, and wild-type presupplemented with purified human CETP (24) to make it equivalent activity in human serum. Numbers of adult pairs assayed were: 6 (A), 8 (B, C), and 5 (D). Data represent average and SE. * $P < 0.05$, ** $P < 0.005$.

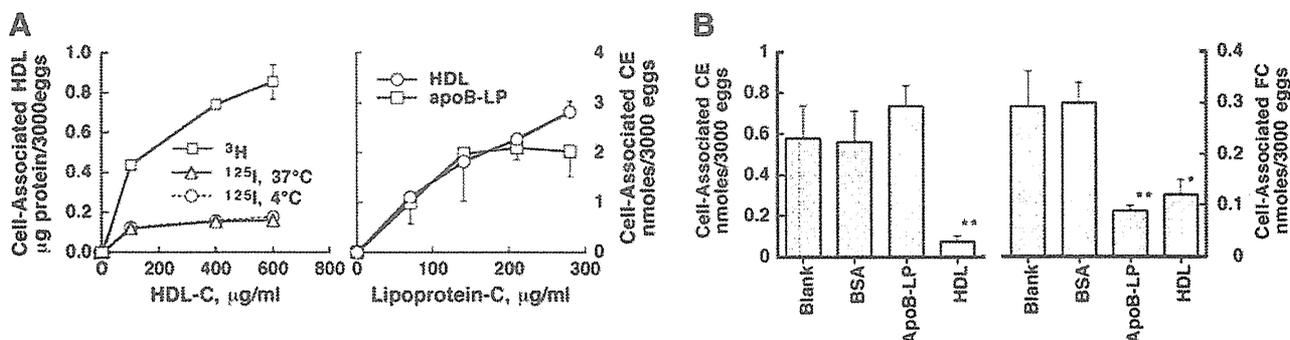


Figure 2. Specific cholesterol uptake from lipoproteins by *S. japonicum* eggs. **A)** Left panel: human HDL was labeled with [^3H]cholesteryl ester (^3H -CE) or ^{125}I . *S. japonicum* eggs (8400/well) were incubated with indicated concentrations of [^3H]-CE-HDL (^3H ; squares) or ^{125}I -HDL (^{125}I ; triangles and circles) at 37°C and 4°C, in 0.5 ml/well RPMI 1640 at 37°C for 20 h. Association of HDL with the eggs was estimated as HDL protein calculated from each specific activity (694.2 dpm/ μg protein for [^3H], 53,380 cpm/ μg protein for ^{125}I). Specific association of [^3H] was assessed as difference between the results at 37 and 4°C. Right panel: human HDL and apoB-lipoprotein (apoB-LP, as $d > 1.063$) were double-labeled with [^3H]-CE [^{14}C]cholesterol as described in Materials and Methods. *S. japonicum* eggs (3000/well) were incubated as in left panel, with the labeled HDL (circles) or apoB-LP (squares). Specific active uptake of CE (cell-associated CE) was determined as difference between the specific uptake values at 37 and 4°C. Horizontal axes indicate lipoprotein cholesterol (C). **B)** Selective uptake of cholesterol by *S. japonicum* eggs from HDL. HDL was double-labeled with [^3H]-CE and [^{14}C]cholesterol, and incubated with the *S. japonicum* eggs (58 μg HDL cholesterol for 3000 eggs/ml medium/well) in the presence of 10 vol of nonlabeled HDL, nonlabeled LDL, or 1 $\mu\text{g/ml}$ BSA. Uptake of CE (cell-associated CE, right panel) or cholesterol (cell-associated FC, right panel) was calculated as the difference between the specific values at 37 and 4°C. Data represent average and SE of the triplicate assay. * $P < 0.05$, ** $P < 0.01$ vs. blank.

mRNA was searched by using the 489-bp probe derived from the cDNA of Sj-Ts2 protein that has one of the CD36 domains (671 bp was submitted to Genbank; see above). The mRNA representing a CD36 family protein was identified in *S. japonicum* adult (Fig. 3A). The approximate size was 1.8 kb, longer than that previously reported for Sj-Ts2 (671 bp). GAPDH mRNA was detected as a 1.2-kb band, consistent with the size of 1148 bp reported for *S. japonicum*.

Screening of a CD36-related gene from the cDNA libraries of *S. japonicum* adults and eggs

From the *S. japonicum* adult cDNA library, Sj-Ts2-containing cDNAs of variable sizes longer than 1 kb were obtained as inserts of $^{32}\text{P}^+$ clones. These cDNA fragments all seemed to be derived by single transcription, including the sequence of the reported Sj-Ts2 protein. Inserts of 8 clones showed >1068 bp elongated from the 5' terminus, and one showed ~100 bp elongated from the 3' terminus of Sj-Ts2 protein. By 5'-RACE analysis, the start codon downstream of a stop codon was detected, and the sequence of the final full-length (1880-bp) original mRNA was determined and deduced to 506 amino acid residues (Supplemental Fig. S1). On the other hand, more than half of 26 inserts of positive clones derived from the egg cDNA library had a deletion of 68-bp nucleotides at positions 98–165 within the coding region. However, the inserts of remaining clones had no deletion and identical sequences with the nucleotide, beginning at position 14 of adult mRNA. These findings indicate that alternative splicing of this mRNA may occur. The size of the PCR product of 5' fragment with 5' primer and GSP2 with the first-strand cDNA derived from total RNA as template was similar (467 bp) between eggs and adults (Fig.

3B), so that the CD36RP mRNA is likely to be expressed in eggs, though the level seems lower (Fig. 3C).

The amino acid sequence indicated that the protein sequence belongs to the CD36 family. It had two transmembrane regions by PHD analysis (23), 15 N-glycosylation sites by PROSITE motif analysis, and CD36 domains by conserved domain (CD) search analysis (Supplemental Figs. S1 and S2). Blastp analysis revealed identity with high score to SRC1_RAT (SR-B1) (48%; E score 8e-51) and with CD36_RAT (49%; E score 5e-45) and SRC_HUMAN (CLA-1) (49%; E score 2e-52). By multialignment (pfam01130) of 32 CD36 family proteins from a variety of organisms by CDD, the highly conserved 4 Cys and 4 Pro residues were demonstrated in domains from IPB002159D to IPB002159F in CD36 family proteins, including this CD36RP (Supplemental Fig. S2, boxed). Three N-glycosylation sites (aa 97, 205, and 248) identified among the 15 candidate glutamines in CD36RP were conserved in mammalian SR-B1 and CD36 (not shown). It had 15 nucleotide polymorphism sites in the coding region identified during screening, resulting in 11 amino acid substitutions.

Expression of recombinant CD36RP

Recombinant GST-fused full size CD36RP (rCD36RP) expressed in *E. coli* and isolated as GSH-Sepharose gel-bound protein was shown as an 82-kDa band by Western blotting, by using anti-GST and anti-Ex160 antibodies (Fig. 4). His-tagged extracellular half size CD36RP (Ex160) was expressed and shown as a 25 kDa polypeptide by Western blotting with anti-RGS-His antibody and antibody was raised against this peptide. After treatment of the GSH-gel-bound rCD36RP with protease, the anti-Ex160 $^+$ band of the apparent molec-

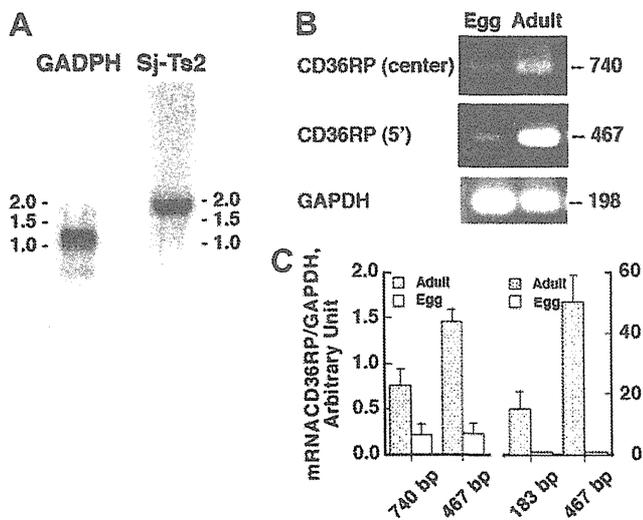


Figure 3. Expression of a CD36-like gene in *S. japonicum*. A) Northern blot hybridization of total RNA isolated from adult *S. japonicum*. Total RNA was subjected to electrophoresis in 1.0% agarose-formaldehyde gel, transferred to nylon membrane, and hybridized with the ³²P-labeled 489- and 198-bp oligonucleotide probes corresponding to Sj-Ts2 and *S. japonicum* GAPDH cDNA, respectively, as described in Materials and Methods. B) Expression of CD36RP mRNA in the eggs and adults of *S. japonicum*. RT-PCR of total RNA was performed for both in the same concentration by using the 5' primer and GSP2 primer for CD36RP (5'), 467 bp; the center primers (L1 and R3) for CD36RP (center), 740 bp; and primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Size of the PCR products is shown. C) Quantitative data of conventional RT-PCR (left panel) and results of quantitative RT-PCR (right panel) performed by using primers L1 and GSP2 (183 bp) and the same primers for CD36RP (5') (467 bp).

ular mass of ~60 kDa (Fig. 4, arrow) was detected in the supernatant, which had no reactivity to anti-GST antibody, indicating the cleavage of GST of 22 kDa. The anti-Ex160 antibody showed stronger reactivity for recognition of CD36RP in *S. japonicum* adults and *S. japonicum* eggs than anti-P 331–348. Ex160 peptide was, however, hardly soluble in aqueous solution without detergents to perform lipoprotein-binding experiments in nondenaturing PAGE. Therefore, a shorter recombinant peptide Ex121, trimmed at the C terminus of Ex160, was expressed in *E. coli* as a GST-fusion protein. Free Ex121 released from GSH Sepharose gel after cleavage of GST (Fig. 4) was able to enter into the gel in nondenaturing PAGE. This solution was used for binding to lipoproteins.

Characterization of *S. japonicum* CD36RP

The particulate and cytosol fractions were prepared from *S. japonicum* adults and mouse liver, and Western blotting analysis was carried out in 10% SDS-PAGE. CD36RP and SR-BI were detected as 82- and 85-kDa bands, respectively, only in the particulate fraction (Fig. 5) and not in the cytosol fraction. After treatment with N-glycanase, the size of CD36RP in the *S. japonicum* adult particulate fractions decreased from 82 to 62 kDa, probed by anti-Ex160 antibody, indicating CD36RP

glycosylation (Fig. 5). By similar treatment of the mouse liver particulate fractions, deglycosylated SR-BI appeared as a 60-kDa band by anti-SR-BI antibody. Murine SR-BI was reported to have 11 N-linked glycosylation sites, 2 of which were important for expression in plasma membrane (26). Additional treatment with N-acetylglucosaminidase resulted in no further reduction of the size of CD36RP. The EndoH treatment showed similar results. The main band in the *S. japonicum* egg particulate fractions was broader around 82 kDa, with a minor band of 62 kDa. Treatment with EndoH seemed to cause no apparent change of the bands, giving inconclusive results as to whether CD36RP is glycosylated and the experimental condition is good for deglycosylation in the eggs.

Lipoprotein binding of the recombinant peptide, Ex121

The water-soluble extracellular domain peptide Ex121 was incubated with HDL and LDL and subsequently analyzed by density-gradient nondenaturing PAGE. The Ex121 peptide was detected by immunoblotting with anti-P 331–348. The position of Ex121 was shifted from that of free Ex121 to the position corresponding to HDL, assured by anti-apoA-I antibody and by HDL samples prestained with Sudan black B (not shown and Fig. 6A). Binding of Ex121 to HDL increased and free Ex121 decreased as the concentration of HDL increased. HDL isolated from the CETP-deficient human serum showed less binding of Ex121 than normal HDL (Fig. 6B, left panel). Ex121 also seemed to bind to LDL but to a much lesser extent, and the Ex121⁺ band was at the position a little smaller than regular LDL parti-

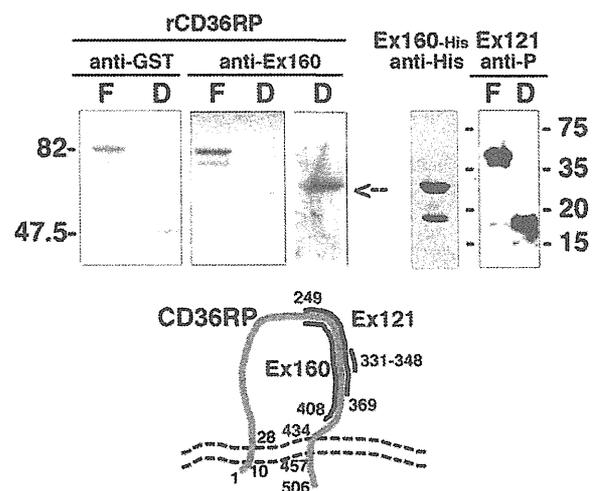


Figure 4. SDS-PAGE of recombinant CD36RP. Full-length CD36RP (rCD36RP) and the extracellular region peptide, Ex121, Gly249-Tyr369, were expressed in *E. coli* as the GST-fusion proteins. Digested product (D) was obtained after treatment of the GST-fusion protein (F) by precision protease, as described in Materials and Methods (arrow). Anti-Ex160 and anti-GST antibodies were used for detection of GST-free rCD36RP (D) and GST-fusion protein (F), and anti-peptide 331–348 (anti-P) for Ex121. Ex160-His peptide was detected by anti-His antibody. Bottom panel: predicted topology of CD36RP and positions of the peptides used.

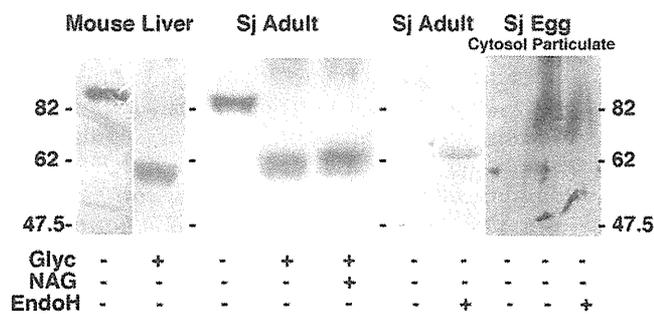


Figure 5. Deglycosylation of Sj CD36RP in the particulate fractions from *S. japonicum* adults. Particulate fractions (48 μ g protein of *S. japonicum* or 100 μ g protein of mouse liver) were treated with or without 10 mU *N*-glycanase in 50 μ l reaction mixture overnight at 37°C, as described in Materials and Methods. Reaction mixtures without enzyme (-), with glycanase (Glyc), or with glycanase plus 35 mU *N*-acetyl glucosaminidase (Glyc, NAG) were analyzed in SDS-PAGE and Western blotting carried out by using anti-Ex160 or anti-SR-BI antibody. Particulate fraction of *S. japonicum* eggs was similarly treated with (+) or without (-) 10 mU EndoH, and CD36RP was detected with anti-P.

cles. When HDL is added, EX121 interacted with LDL is likely to be transferred to HDL, but the Ex121-HDL complex seemed shifted to a position of higher molecular mass (Fig. 6B, right panel). These results suggest that an extracellular fragment containing the conserved Cys and Prorich domain of *S. japonicum* CD36RP binds to HDL particles (27). This association seems to be selective.

Effect of the anti-Ex160 antibody on HDL-cholesteryl ester uptake and maturation of the eggs

The antibody against the extracellular domain peptide Ex160 was examined for CD36RP activity for HDL cholesteryl ester uptake and egg maturation (Fig. 7). The effect of the antibody was observed on HDL-cholesteryl ester uptake by the eggs. The antibody suppressed the cholesteryl ester uptake at 37°C but not at 4°C, so that significant suppression was for the active uptake shown as NET uptake in Fig. 7A. The antibody was added to the egg maturation assay system of Fig. 1, except that a pair of the parent adults was cultured for 10 d in the presence of 10% serum in the presence of

Figure 6. Association of recombinant protein Ex121 with HDL. A) Ex121 (35 ng) was incubated with 1–5 μ g protein of HDL in 8 μ l PBS at room temperature for 30 min and at 4°C for 3 h, and then mixed with 6 μ l native sample buffer (62 mM Tris-HCl, pH 6.8; 10% sucrose; and 0.1% BPB). The double samples were subjected to nondenaturing PAGE with 4–20% gradient Tris-Gly gel and detected with anti-P parallel with anti-apoA-I. B) Ex121 (70 ng) was incubated with 30 μ g protein of HDLs obtained from normal subjects (normal 1, normal 2) or CETP-deficient subject (CETP-d), or LDL in 7 μ l PBS (left panel), or with 15 μ g protein of HDL or LDL or mixture of HDL and LDL (right panel) in 12 μ l PBS, at room temperature for 30 min. Western blotting was carried out using anti-P. Arrows indicate positions of HDL prestained by Sudan black.

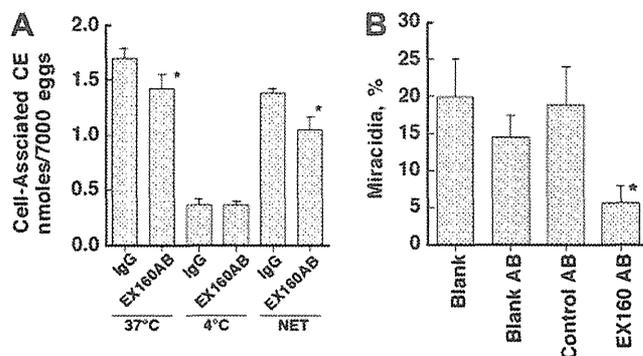
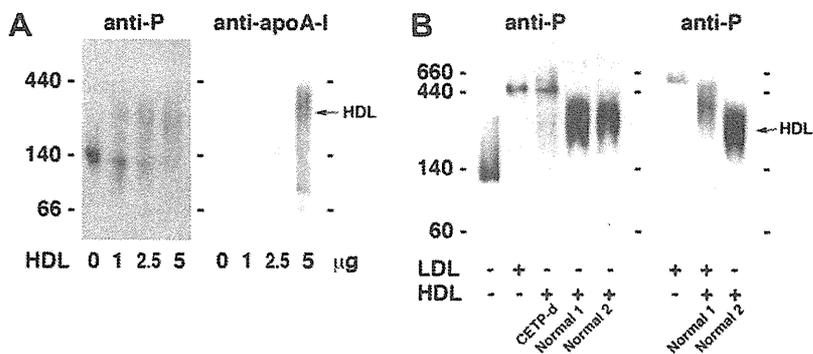


Figure 7. Suppression of HDL-cholesteryl ester (CE) uptake and maturation of the eggs by the antibody against EX160. A) Uptake of HDL-CE was measured in the same system as used in Fig. 2, in the presence of the antibody. B) Maturation of *S. japonicum* eggs was estimated in the same condition as Fig. 1, except for using 5% serum, in the presence of the antibodies. IgG, nonimmune rabbit IgG; EX160AB, antibody against Ex160; blank, with no additional antibody/antisera; blank AB, with 1:100 vol of nonimmune rabbit serum; control AB, with 1:100 vol of rabbit antisera against the intracellular domain peptide of CD36RP (anti-P 331–348). Titer of Ex160 was adjusted to <1/25 of the anti-P antisera. Data represent means \pm SE of $n = 6$ /group. * $P < 0.05$ vs. IgG (A) or all other treatments (B).

the antibody. Maturation of the eggs to miracidia was significantly reduced in the presence of the anti-Ex160 antibody in comparison to control antibody, anti-P 331–348, or blank (Fig. 7B).

DISCUSSION

Schistosomes are the parasites that finally reside in the blood vessels of the host patients, and in the case of *S. japonicum*, the adult flukes locate to the portal vein and its draining venules. Chronic schistosomiasis causes various pathological problems in the body, not only from the parasites themselves but also the eggs laid in the body. The adult worms lay eggs in the portal vein to be released to the intestinal tract, but many are flushed back to the liver, where they embolize and develop into miracidia, a phenomenon that deals with the morbidity and mortality of hepatic granulomatosis (1–5).

We previously reported that maturation of *S. japonicum* eggs to miracidia requires normal HDL, presumably as a nutrient supplier, but it is insufficient in culture in the presence of the HDL recovered from CETP-deficient patients (10). We confirmed this process in more detail, as presented in Fig. 1. This was consistent with the *in vivo* findings that the egg maturation in the liver and hepatic granulomatosis were less in wild-type mice, which lack endogenous CETP activity, than in the CETP transgenic mice (10). We therefore investigated potential candidate factors to catalyze this reaction. Both schistosome eggs and adults were found to selectively take up cholesteryl ester from HDL particles. As this type of reaction is known to be carried out by CD36-like proteins, including SR-BI, in many animals (25–27), we searched for schistosome genes homologous to CD36. We used probes with the sequence consisting of coding region of Sj-Ts2 protein (Genbank AF291715), which appeared to have one of the CD36 domains by Prodom analysis, for Northern blot analysis screening of the *S. japonicum* adult cDNA library, and an identified positive band of mRNA of ~1.8 kb in length. Based on this information, we cloned from the *S. japonicum* adult cDNA library a cDNA of 1880 bp that was deduced to encode 506 aa, and have termed this CD36RP. The transcript encoding this protein was also expressed by eggs of *S. japonicum*. Sequence and structural analyses showed clear relatedness to other CD36 family proteins. The extracellular domain peptide of CD36RP demonstrated selective binding to normal HDL but markedly reduced interaction with HDL from CETP-deficient patients. Finally, the antibody against the extracellular domain of CD36RP suppressed HDL-cholesteryl ester uptake and maturation of the eggs *in vitro*.

Based on these new findings, we now propose that CD36RP is a lead candidate for a mediator of selective uptake of cholesteryl ester from HDL by *S. japonicum* necessary for egg maturation to miracidia. It is instructive that preexposure of the adult schistosomes to standard (wild-type) HDL is sufficient for the eggs to mature, perhaps because the vitelline of the egg were preformed adequately in such a condition. In contrast, even the eggs with inadequate vitelline (yolk) provisions may mature provided that normal HDL is supplied after the eggs are laid (*i.e.*, released from the female schistosome into the culture medium). The data we present here support the view that absence of normal HDL retards maturation of the *S. japonicum* eggs in the host liver and, accordingly, prevents hepatic granulomatosis, in a situation such as CETP deficiency where abnormal large HDL does not efficiently bind CD36RP (Fig. 6B). This may be one of the reasons why the prevalence of CETP deficiency is so high in the Far East (11–14) where schistosomiasis japonica has been and/or remains common in rural or underdeveloped areas. If this hypothesis were valid, CETP inhibitors could be useful to prevent hepatic granulomatosis in schistosomiasis. This would be reminiscent of the selective advantage that hemoglobinopathies confer against malaria (*e.g.*, see ref. 28 and references therein).

The question may remain whether cholesterol is a specific nutrient for this reaction. Although triglyceride

is a minor component of HDL core, it may still contribute to maturation of the *S. japonicum* eggs and HDL in CETP deficiency is characterized as low content of triglyceride (29,30). The particular CETP-deficient HDL used in the experiment, however, contained triglyceride as 1.6% of cholesteryl ester and 3.3% of phospholipid as mass, while normal HDL contained it as 0.5–4% and 1–6%, respectively, depending on plasma VLDL concentration. Therefore, contribution of triglyceride is less likely.

We have attempted at length to demonstrate functional alteration of cholesteryl ester uptake by adults or eggs of *S. japonicum* by manipulating expression of the whole CD36RP protein. However, neither functional expression by transfection of full-length CD36RP nor knockdown/knockout of the gene in the parasite cells was successful so far, seemingly because of various profound technical problems, including expressing the parasite genes in cells or cell lines from other species. Therefore, we do not have direct evidence that CD36RP mediates the selective uptake of cholesteryl ester by schistosome adults or eggs. However, structural similarity of CD36RP to CLA1 or SR-BI that mediate cholesteryl ester uptake from HDL in the cells of human and rodents, respectively, can be extrapolated to the functional similarity. Furthermore, the extracellular domain of CD36RP indeed demonstrated selective binding to HDL, and its antibody suppressed HDL cholesteryl uptake and maturation of the eggs in culture. Thus, it is not unreasonable to speculate that CD36RP is a strong candidate for a mediator of HDL cholesteryl ester uptake by the adults and eggs of *S. japonicum*, and therefore a key molecule for maturation of the egg to the miracidium. This means that CD36RP, as well as host plasma HDL, is a key protein for hepatic granulomatosis in *S. japonicum* infection that can represent a fatal pathological process in infected persons.

Clearly it will be necessary to demonstrate more direct evidence for CD36RP to catalyze selective cholesteryl ester uptake before this schistosome glycoprotein can be definitively ascribed a role as the mediator of the reaction. Technical difficulties remain to be overcome in order to accomplish the necessary manipulations, such as functional transfection and expression of the gene or knockdown of the gene to down-regulate the reaction. However, given recent advances with transgenesis approaches in schistosomes and other parasitic helminths (31–33), it is feasible that informative functional genetics approaches may soon allow definitive assignment or not of a physiological role for schistosome CD36RP in selective uptake of cholesteryl ester from host HDL. EJ

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Complete mitochondrial genomes of *Diplogonoporus balaenopterae* and *Diplogonoporus grandis* (Cestoda: Diphylobothriidae) and clarification of their taxonomic relationships[☆]

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ABSTRACT

Although the diplogonadic human tapeworm, *Diplogonoporus grandis*, has long been considered to be a synonym of the whale tapeworm, *Diplogonoporus balaenopterae*, the identity of the both species at the complete mitochondrial genomes and nuclear DNA levels has been not sufficiently undertaken to date. In the present study, to clarify the taxonomic relationships between *D. balaenopterae* and *D. grandis* at the molecular level, the complete mitochondrial genomes of both species were sequenced and compared. In addition, the genetic variation in the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) and the nuclear internal transcribed spacer-1 (ITS-1) region of the ribosomal RNA gene were examined. The complete mitochondrial genomes of *D. balaenopterae* and *D. grandis* consisted of 13,724 bp and 13,725 bp, respectively. These mitochondrial genomes contained 12 protein-coding, 22 transfer RNA and 2 ribosomal RNA genes and two longer non-coding regions. Except for *Hymenolepis diminuta*, the genomic organization in both species was essentially identical to that in other cestode genomes examined to date. However, differences were observed between *Diplogonoporus* and *Diphylobothrium* species in abbreviated stop codons, sequences and the number of repeat units in the 2nd non-coding regions. The genetic differences observed in the mitochondrial genomes, *cox1* and ITS-1 regions of both species were considered typical of intraspecific variation. In conclusion, *D. balaenopterae* is a taxonomically valid species and *D. grandis* is a junior synonym of *D. balaenopterae* based on the zoological nomenclature. Further, molecular-phylogenetic analysis confirmed that *D. balaenopterae* is more closely related to *Diphylobothrium stemmacephalum*, the type-species of the genus *Diphylobothrium*, and the taxonomical validity of the genera *Diplogonoporus* and *Diphylobothrium* was also discussed.

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1. Introduction

Broad tapeworms belonging to the genus *Diplogonoporus* Lönnberg, 1892, are characterized by having a double set of genitalia in a single proglottid, distinguishing them from *Tetragonoporus* Skriabin, 1961, *Hexagonoporus* Gubanov in Delyamure, 1955, and *Polygonoporus* Skriabin, 1967, which all possess multiple gonads in a single proglottid,

and many members of *Diphylobothrium* Cobbold, 1858, which usually have only one set of gonads per proglottid [1]. *Diplogonoporus balaenopterae* Lönnberg, 1892, infects the small intestine of whales, such as the minke whale (*Balaenoptera acutorostrata*, Balaenopteridae, Cetacea), sei whale (*Balaenoptera borealis*, Balaenopteridae, Cetacea) and humpback whale (*Megaptera novaeangliae*, Balaenopteridae, Cetacea) [1]. In contrast, *Diplogonoporus grandis* Lühe, 1899, which causes diplogonoporiosis in humans, has been regarded as a synonym of *D. balaenopterae*, based on adult tapeworm morphology [2–5], characteristics of larval coracidia and proceroids [6,7], and protein profiles [8]. Nevertheless, *D. grandis* has been referred to as the causative agent of human diplogonoporiosis, especially in Japan [9,10].

Diplogonoporiosis cases in humans have been found almost exclusively in Japan where more than 200 cases have been reported over the last 100 years [11]. Outside Japan, a total of 3 cases have been reported in Chile [12], Korea [13] and Spain [14]. In the latter two cases, the etiologic agents were identified as *D. balaenopterae* based on proglottid morphology. While the complete life cycles of these tapeworms have not yet been elucidated, Japanese anchovy or “shir-asu” (*Engraulis japonica*, Engraulidae, Clupeiformes), Japanese sardine

Abbreviations: *atp6*, ATPase subunit 6 gene; *cob*, cytochrome *b* gene; *cox1–cox3*, cytochrome *c* oxidase subunits 1–3 genes; *nad1–nad6*, NADH dehydrogenase subunits 1–6 genes; *nad4L*, NADH dehydrogenase 4 large subunit gene; *rnl*, ribosomal RNA large subunit gene; *rns*, ribosomal RNA small subunit gene; *trn*, transfer RNA genes; PCR, polymerase chain reaction.

[☆] Nucleotide sequences of the *D. balaenopterae* and *D. grandis* mitochondrial genomes reported in the present paper are deposited at the DDBJ/GenBank databases under accession numbers AB425839 and AB425840, respectively. AB355622–AB355626, AB355628, AB355629, AB474567, and AB474568 are the accession numbers for the *cox1* gene and AB449346–AB449356, AB474569, and AB474570 are the accession numbers for the ITS-1 regions.

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Table 1
Diplogonoporus isolates examined in the present study.

Sample no.	Year collected	Locality collected (latitude/longitude)	Host animals	DBJ/GenBank accession numbers	
				<i>cox1</i>	ITS-1
No. 1	1997	Western North Pacific Ocean (N37/E160)	<i>Balaenoptera acutorostrata</i> (minke whale)	AB355622	AB449346
No. 2 ^a	1997	Western North Pacific Ocean (N37/E163)	<i>B. acutorostrata</i>	AB355623	AB449347
No. 3	1997	Western North Pacific Ocean (N39/E161)	<i>B. acutorostrata</i>	AB355624	AB449342
No. 4	1997	Western North Pacific Ocean (N39/E161)	<i>B. acutorostrata</i>	AB355625	AB449349–AB449352
No. 5	1997	Western North Pacific Ocean (N39/E158)	<i>B. acutorostrata</i>	AB355626	AB449353
No. 6	2002	Western North Pacific Ocean	<i>Balaenoptera borealis</i> (sei whale)	AB474567	AB474569
No. 7	2002	Western North Pacific Ocean	<i>B. borealis</i>	AB474568	AB474570
No. 8 ^a	2004	Tokyo, Japan	<i>Homo sapiens</i> (58-year-old Japanese man)	AB355628	AB449354
No. 9	2006	Hamamatsu, Shizuoka, Japan	<i>H. sapiens</i> (58-year-old Japanese man)	AB355629	AB449355, AB449356

^a *Diplogonoporus* isolates used for the complete mitochondrial genome analysis.

(*Sardinops melanostictus*, Clupeidae, Clupeiformes) [9] and skipjack tuna (*Katsuwonus pelamis*, Scombridae, Perciformes) have been suspected to be the most likely sources of infection in humans [10].

To assess the phylogenetic relationships among eucestodes, genes such as the ribosomal RNA large subunit (28S rRNA) and small subunit (12S rRNA) genes [15–20], *cox1* and *nad3*[21], elongation factor-1 alpha gene [16], the internal transcribed spacer (ITS) regions [22,23] and the 18S rRNA gene [19,24] have been used. With regard to *Diplogonoporus* isolates, preliminary DNA analysis using *cox1* recently supported the assignment of *D. grandis* as a synonym of *D. balaenopterae* [25,26]. Genetic analysis of *Diplogonoporus* isolates from clinical cases has recently revealed a close relationship between *Diplogonoporus* and *Diphyllobothrium stemmacephalum*[24].

Thus, in the present study, the complete mitochondrial genomes of both species were sequenced and compared in order to clarify the molecular–taxonomic relationship between *D. balaenopterae* and *D. grandis*. In addition, the genetic variation within the *cox1* and ITS-1 regions was examined using nine *Diplogonoporus* isolates obtained from whales and humans. The need for a revision of the taxonomic affiliation of the genera *Diplogonoporus* and *Diphyllobothrium* is also discussed.

2. Materials and methods

2.1. *Diplogonoporus* tapeworms examined in the present study

The *Diplogonoporus* tapeworm specimens examined in this study are listed in Table 1. Minke whale and sei whale were taken in the Western North Pacific Ocean with special permission from The Institute of Cetacean Research, Japan. The seven mature *Diplogonoporus* tapeworms collected from the small intestines of the whales were identified as *D. balaenopterae* based on morphological characters (Nos. 1–7 in Table 1). Two diplogonadic tapeworms, one immature tapeworm lacking a scolex (No. 8) and a mature tapeworm with a scolex (No. 9), were obtained from two Japanese patients; these samples were identified morphologically as *D. grandis*. The tapeworms were rinsed thoroughly in phosphate-buffered saline after collection and preserved in 80% ethanol, except for two specimens (Nos. 6 and 9) which were fixed in 10% formalin.

2.2. DNA extraction, PCR amplification and DNA sequencing

DNA was extracted from the ethanol-fixed proglottids using a DNeasy Blood & Tissue kit (Qiagen, Germany) according to the manufacturer's instructions. The formalin-fixed specimens were embedded in paraffin and DNA was efficiently extracted from the unstained, 10 µm-thick sections using a DEXPAT kit (Takara Bio Inc., Japan) as described previously [26,27]. For analysis of the complete mitochondrial genome, DNA was extracted from two representative tapeworms fixed in ethanol (Nos. 2 and 8 in Table 1). Amplification of the mitochondrial genomes was performed using 13 primer pairs

(Table 2) designed based on the mitochondrial genomes of *Diphyllobothrium nihonkaiense* (AB268585) and *Diphyllobothrium latum* (AB269325). The PCR consisted of an initial denaturation step of 98 °C for 30 s, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s, with a final extension cycle of 72 °C for 5 min. Samples were amplified in a final reaction volume of 50 µL with *Ex Taq* DNA polymerase (Hot Start version, Takara Bio Inc., Japan).

For the polymorphism analysis of the *cox1* and ITS-1 regions of the ethanol-fixed samples (Nos. 1, 3–5 and 7), primer pairs P1/P2 and P28/P31 were designed based on the nucleotide sequences of the *cox1* and ITS-1 regions in *Diplogonoporus* and *Diphyllobothrium*, respectively (Table 3). For the formalin-fixed samples (Nos. 6 and 9), short and overlapping DNA fragments were amplified using primer pairs P3–P27, and P28/P29 and P30/P31 (Table 3) for the *cox1* and the ITS-1 regions, respectively. High fidelity *KOD FX* DNA polymerase (Toyobo, Japan) was occasionally used for DNA samples extracted from formalin-fixed materials. PCR performed using *KOD FX* DNA polymerase employed an initial denaturation step of 94 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s and a final cycle of 72 °C for 5 min.

Amplicons confirmed by agarose gel or capillary electrophoresis (HAD-GT12, eGene Inc., LA) were purified using a NucleoSpin Extract II kit (Macherey-Nagel, Germany) and used as templates for direct DNA sequencing. Samples for DNA sequencing were prepared using

Table 2
Oligonucleotide primers used for amplification of mitochondrial genomes.

Primer pairs	Nucleotide sequences (5' to 3')
P1 atp6/F1	ATGATCTTCAGTGGTTATTCAAGTT
P2 nad1/R25	CACCTGTTAAAAACATAAAAAATCAT
P3 trnA/F	ACAGAATACTGGGTTTGGCTCTCAG
P4 nad3/R60	AAATGATATGACTATAAAACAACJAA
P5 nad3/F1	ATGTTAGCTTATTTTTGGTGG
P6 rnl/R250	CTATACACATTTACITGTCTCTC
P7 trnT/F38	CAGGGGTGGGTTTACTCTTTGGGCCT
P8 trnC/R25	TACTAAGACCAAAGGCAATAGACTT
P9 rnl/F451	CATATTATAAATTTATATGTAGG
P10 trnC/R25	TACTAAGACCAAAGGCAATAGACTT
P11 rnl/F880	TGAGGTGAGTTAAGACCGCGTGAG
P12 rnS/R245	ATTTCACCTACTCTACCTTTACCT
P13 trnC/F40	GTGAATATTGTTTATTCTAGGCTTT
P14 cox2/R25	CGTAGTACAGCAAAGAAAATTCAT
P15 rnS/F570	GTAACAAGGTAGCCAGATGAATC
P16 trnE/R25	TTATGCTCCAATACAACAAACACAGG
P17 cox2/F525	GGTGGGTACCEGTTATATGCCIAATA
P18 nad/R761	CAAGTGGATATGGCAACTATCTCT
P19 nad5/F565	CTACCCCTGTGATGTTCTTAGTACA
P20 cox3/R205	TACCAAAGGCTAAAACITCIAAG
P21 trnG/F40	GTGGGGATCTAATGGTTTATAGATAA
P22 trnH/R25	GCCAGTTTAAATAACCTATCAGTAA
P23 cox3/F444	GGTCTAGATTTTATGCTAGTTGT
P24 nad4/R 325	ACAGAGGTAACATGGATAGCTCATA
P25 nad4/F1	ATGAGACTGTACAAAATTTAGAT
P26 atp/R25	AATCTTGAATAACCAATAAGATCAT

Table 3
Oligonucleotide primers used for the amplification of *cox1* genes and ITS-1 regions.

Primers	Nucleotide sequence (5' to 3')
P1 nad3/F120	CGAGTGTGGTTTTAGATCTTCTTCA
P2 rnl/R250	CTATACACATTTACTTGATCTCCTC
P3 nad3/F120	CGAGTGTGGTTTTAGATCTTCTTCA
P4 <i>cox1</i> /R125	ATACGTATCATAACACTAAGGCTCA
P5 <i>cox1</i> /F1	ATGATAATCTTAAAGTTTTAGTT
P6 <i>cox1</i> /R225	GGGCATCAAAAAAGAATATCATT
P7 <i>cox1</i> /F101	TGAGCCTTAGTGTATGATACGTAT
P8 <i>cox1</i> /R325	ATAAAATCMGCATTAMGCTTT
P9 <i>cox1</i> /F201	AATGATATTCITTTTTTGTATGCC
P10 <i>cox1</i> /R425	TTTCTATCCCTAAAAAGAGCAGAAG
P11 <i>cox1</i> /F301	AAAGCTTTAAGTGCTTGATTTGTTAT
P12 <i>cox1</i> /R525	ATCAAAAAGCTGTACAGGGTA
P13 <i>cox1</i> /F501	TACCTGTACACAGCTTTTGTGAT
P14 <i>cox1</i> /R725	TCAGGATGACCAAAAAATC.AAAACA
P15 <i>cox1</i> /F601	GTTTTAGTCTGCTATTACMTGT
P16 <i>cox1</i> /R825	AAATAATAMCCATAAAAAATC.AAAA
P17 <i>cox1</i> /F701	TGTTTTGATTTTTGGTCATCCTGA
P18 <i>cox1</i> /R925	TAATGACTAAAAAACAGTGT
P19 <i>cox1</i> /F901	ACAGCTGTTTTTTAGTTCAGTTA
P20 <i>cox1</i> /R1125	AACCACAAATCAAGTATCATGCTTT
P21 <i>cox1</i> /F1080	TGCTTGTGTTCTTGATAATTTTTG
P22 <i>cox1</i> /R1309	AACCCATATACCAAGTAAATGCAT
P23 <i>cox1</i> /F1230	ATTGCAGTGTATTGTATAGTGTCT
P24 <i>cox1</i> /R1435	TAAGTAGAGACTCCACAAAATAAAC
P25 <i>cox1</i> /F1401	TTTTTTGTGTTATTTTGTGGGAG
P26 <i>cox1</i> /R1566	CTATAAGGCCAACATATATCTACAAA
P27 <i>trnT</i> /R23	ACAAAACCAAGTATTCTAATTTAA
P28 ITS-1/F1	ACCTGCCGGAAGGATCATTACAGCTT
P29 ITS-1/R325	ACACGACGCCCTCGAGTCTTACGCTT
P30 ITS-1/F301	AGGCCGTAAGACTCGAGGCGTCTGT
P31 ITS-1/R625	AATTCACACAGTTGGCTCGCTCTTC

an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Inc., CA) and nucleotide sequences were determined by primer walking using an ABI PRISM 3100-*Advant* Genetic Analyzer (Applied Biosystems Inc.).

2.3. Data processing and phylogenetic analysis

A number of models for genetic distance analysis and estimation of phylogenetic trees have been proposed and differ in terms of the parameters used to describe the rates of nucleotide substitution during evolution [28]. In this paper, genetic distance of 36 genes encoded in the mitochondrial genomes between *D. balaenopterae* and *D. grandis*, genetic variation of *cox1* and ITS-1 regions among *Diplogonoporus* isolates was estimated using Kimura's 2-parameter (K2P). The model distinguishes between transitional and transversal substitutions of the nucleotides and assumes that all of the bases are equally frequent [29]. The rate of transitional substitution is often higher than that of transversal substitution, especially in animal mitochondrial DNA, and the model was considered to be suitable for estimating genetic distance of mitochondrial DNA [30]. Genetic distance was calculated by the setting of substitution included transitions and transversions, and the rates among sites (gamma parameter = 1).

Phylogenetic analyses of *Diplogonoporus* and *Diphyllobothrium* were performed using nucleotide sequences of *cox1* from 12 *Diphyllobothriidae* taxa (DQ768191, AB369249, AB015753, AB517949, EU241311, EU241317, EU241308, FM209182, AB510496, AM412738, AB268585, AB364645), *Bothriocephalus acheilognathi* (*Bothriocephalidae*, *Bothriocephalidea*, HM439384) and *Taenia solium* (*Taeniidae*, *Cyclophyllidea*, AB516957) were used for comparisons. The nucleotide sequence data were initially aligned with the ClustalW program (ver. 1.83, <http://www.clustalw.ddbj.nig.ac.jp>). The maximum likelihood analysis (ML) and Bayesian inference (BI) were employed for phylogenetic analyses. For the ML, the alignment data were converted to FASTA format using MEGA program (ver. 5.05) and analyzed using Hasegawa-Kishino-Yano (HKY) + G model (gamma = 5) which was

selected based on Bayesian Information Criterion scores using MEGA 5.05.

For BI, the alignment data were converted to NEXUS format using the ClustalX2 (ver. 2.0.12) and Bayesian phylogenetic analysis was performed using MrBayes 3.1.2 (<http://mrbayes.csit.fsu.edu/index.php>). The DNA data were divided into 3 partitions and likelihood setting was set to nst = 6, equivalent to the general time reversible (GTR) model of nucleotide substitution [31]. Markov Chain Monte Carlo analysis was then run on each of the datasets for 10⁶ generations to estimate the posterior probabilities of trees [32]. Phylogenetic trees were rooted with *T. solium* as the outgroup. For the ML analysis, nodal support was assessed by bootstrap resampling (1000 replicates). In BI, clades were considered to have high nodal support if the BI posterior probability was ≥ 0.95.

3. Results and discussion

3.1. Characterization of the complete mitochondrial genomes of *D. balaenopterae* and *D. grandis*

The 5'- and 3'-ends of the protein-coding genes were deduced from the sequences corresponding to the genes in *D. nihonkaiense* and *D. latum*. Using sequence motifs, genes for 2 rRNAs and 22 tRNAs were also annotated in the 5'- and 3'-flanking regions of the open reading frames. In the 2nd non-coding region (NCR2), located between *nad5* and *trnG*, at least three ladder-products were amplified in both species examined (data not shown), with the largest product (320 bp) used to estimate the whole genome size. The complete mitochondrial genome sizes of the isolates No. 2 (*D. balaenopterae*, AB425839) and No. 8 (*D. grandis*, AB425840) were thus estimated to be 13,724 bp and 13,725 bp, respectively.

As shown in Table 4, the mitochondrial genomes consisted of 12 protein-coding genes (*atp6*, *cob*, *cox1-cox3*, *nad1-nad6*), 2 rRNA genes (*rnl*, *rns*), 22 tRNA genes (*trns*), and two longer non-coding regions (NCR1, NCR2). The gene encoding ATPase subunit 8, as well as other flatworm mitochondrial genomes, was absent. Most of the genes were interrupted by several bases and no introns were present in the protein-coding genes. Genes were arranged unidirectionally and exhibited a strong bias toward adenine (A) and thymine (T), with the A + T content reaching 68.7–68.8%. Genomic organization was essentially identical to that reported in other diphyllid and cyclophyllid cestodes to date, except for *Hymenolepis diminuta* [33–37].

Of the 12 protein-coding genes, 11 were initiated by an ATG codon, while *cox3* was initiated by GTG (Table 4). Eleven protein-coding genes were predicted to end with complete stop codons: five (*atp6*, *cox2*, *nad2*, *nad5* and *nad6*) were predicted to terminate with TAA and six (*cob*, *cox1*, *nad1*, *nad3*, *nad4* and *nad4L*) with a TAG codon. However, *cox3* in the *Diplogonoporus* isolates was predicted to end with an abbreviated stop codon, T (Table 4), and a complete stop codon appeared to be missing downstream of *trnH*, suggesting that the abbreviated T has been modified by post-transcriptional polyadenylation. It is not considered unusual to find incomplete termination codons, such as T or TA, in the protein-coding genes of metazoan mitochondrial genomes [38]. Indeed, in the mitochondrial genome sequences of flatworms that have been reported to date, abbreviated stop codons have been identified in *cox3*, *nad1* and *nad3* of *D. nihonkaiense* and *D. latum* [34], and *nad1* in *T. solium* [35].

The NCR2 region is considered to be involved in the replication of mitochondrial DNA [39]. Kim et al. [37] reported that size variation in the NCR2 region is largely due to differences in the number of identical 36-nucleotide repeat sequence units, and that these differences vary according to geographic location in *D. latum* (4–6 repeats) and *D. nihonkaiense* (4–7 repeats). In the NCR2 region of *Diplogonoporus* isolates from whales and humans, at least 8 identical repeat units consisting of 33 nucleotides (5'-TTAGGGATGTGTAGTATATTCTCTAAATTG-3')

Table 4Location of genes and non-coding regions (NCR) in the mitochondrial genomes of *D. balaenopterae* and *D. grandis*.

Genes and NCRs	<i>D. balaenopterae</i> (13,724 bp)			<i>D. grandis</i> (13,725 bp)		
	Locations	Length (bp)	Start–Stop codons	Locations	Length (bp)	Start–Stop codons
<i>trnY</i>	1–66	66		1–66	66	
1st NCR	67–291	225		67–292	226	
<i>trnL1</i> (CUN)	292–358	67		293–359	67	
<i>trnS2</i> (UCN)	380–445	66		379–444	66	
<i>trnL2</i> (UUR)	450–513	64		449–512	64	
<i>trnR</i>	514–568	55		513–567	55	
<i>nad5</i>	572–2140	1569	ATG-TAA	571–2139	1569	ATG-TAA
2nd NCR	2141–2460	320		2140–2459	320	
<i>trnG</i>	2461–2528	68		2460–2527	68	
<i>cox3</i>	2532–3174	643	GTG-T ^a	2531–3173	643	GTG-T ^a
<i>trnH</i>	3175–3238	64		3174–3237	64	
<i>cob</i>	3242–4348	1107	ATG-TAG	3241–4347	1107	ATG-TAG
<i>nad4L</i>	4350–4610	261	ATG-TAG	4349–4609	261	ATG-TAG
<i>nad4</i>	4571–5821	1251	ATG-TAG	4570–5820	1251	ATG-TAG
<i>trnQ</i>	5822–5884	63		5821–5883	63	
<i>trnF</i>	5881–5945	65		5880–5944	65	
<i>trnM</i>	5942–6007	66		5941–6006	66	
<i>atp6</i>	6011–6520	510	ATG-TAA	6010–6519	510	ATG-TAA
<i>nad2</i>	6523–7401	879	ATG-TM	6522–7400	879	ATG-TM
<i>trnV</i>	7404–7468	65		7403–7467	65	
<i>trnA</i>	7471–7533	63		7470–7532	63	
<i>trnD</i>	7537–7598	62		7536–7597	62	
<i>nad1</i>	7599–8489	891	ATG-TAG	7598–8488	891	ATG-TAG
<i>trnN</i>	8489–8553	65		8488–8552	65	
<i>trnP</i>	8567–8629	63		8566–8628	63	
<i>trnI</i>	8640–8702	63		8639–8701	63	
<i>trnK</i>	8709–8773	65		8708–8772	65	
<i>nad3</i>	8775–9131	357	ATG-TAG	8774–9130	357	ATG-TAG
<i>trnS1</i> (AGN)	9121–9179	59		9120–9178	59	
<i>trnW</i>	9181–9243	63		9180–9242	63	
<i>cox1</i>	9252–10,817	1566	ATG-TAG	9251–10,816	1566	ATG-TAG
<i>trnT</i>	10,808–10,869	62		10,807–10,868	62	
<i>ml</i>	10,870–11,831	962		10,869–11,831	963	
<i>trnC</i>	11,832–11,895	64		11,832–11,895	64	
<i>ms</i>	11,896–12,625	730		11,896–12,626	731	
<i>cox2</i>	12,626–13,195	570	ATG-TAA	12,627–13,196	570	ATG-TAA
<i>trnE</i>	13,198–13,266	69		13,199–13,267	69	
<i>nad6</i>	13,263–13,721	459	ATG-TAA	13,264–13,722	459	ATG-TAA

^a Abbreviated stop codon.

were confirmed, but variation in the number of repeat units among geographical isolates of *Diplogonoporus* has not yet been thoroughly investigated.

3.2. Taxonomic status between *D. balaenopterae* and *D. grandis* based on molecular analyses

Genetic distances of 36 genes encoded by the mitochondrial genomes of both species were calculated using K2P model. The genetic distance values between the 12 protein-coding genes were extremely small ($d = 0.0000$ – 0.0054). Similarly, the genetic distances between the *ml* and *ms* genes were low at 0.0012 and 0.0030, respectively. Of the 22 *trns*, 19 had identical sequences and *trnC*, *trnH* and *trnV* were separated by a distance of $d = 0.0158$. By way of comparison, the genetic distance value obtained for the *cox2* in *D. nihonkaiense* and *D. latum*, which are distinct species, was 0.069. Indeed, the value for *cox2* was the lowest genetic distance observed among all of the protein-coding genes of *D. nihonkaiense* and *D. latum* (data not shown), indicating that the differences between *D. balaenopterae* and *D. grandis* were more likely due to intraspecific variation than because the two taxa were separate species.

Fig. 1 is an unrooted neighbor-joining tree showing the genetic relationships among diplogonoporiid isolates estimated using *cox1* (A) and ITS-1 region (B) sequences. The transitional ($A = G, T = C$) and transversal substitutions ($T = G, T = A$) were at 25 and 2 sites (1393 and 1476), respectively, along the 1566-bp *cox1* sequence (data not shown). The genetic distance ranged from 0.0000 to

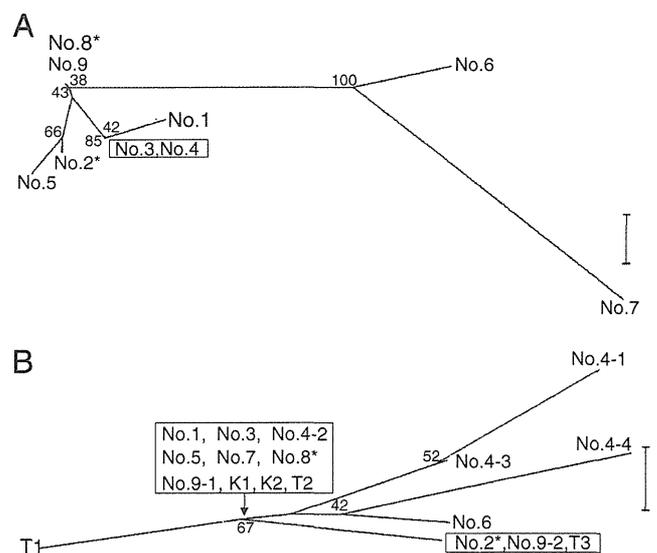


Fig. 1. Unrooted neighbor-joining trees inferred from the sequences of the (A) *cox1* (1566 bp) and (B) ITS-1 region (625 bp) of *Diplogonoporus* isolates. Numbers at the nodes indicate bootstrap values (1000 replicates). Asterisks denote samples used for the complete mitochondrial genome analysis. ITS-1 regions of *D. grandis* isolates K1, K2, T1, T2 and T3 are from accession numbers AB298510, AB298511, AB298512, AB298513 and AB298514, respectively. Bars = 0.001 (A) and 0.0005 (B).

0.0158 (overall mean = 0.0059), which are typical of intraspecific variations. Nucleotide sequences of the ITS-1 region (625 bp) of 2 *Diplogonoporus* isolates (Nos. 4 and 9) were determined using PCR products subcloned into pT7 Blue T-vector (Novagen, USA) because dual peaks at 4 sites were detected by direct DNA sequencing. Polymorphic nucleotides were observed at 4 sites (30, 147, 334 and 548) in *D. balaenopterae* (No. 4) and at position 344 in *D. grandis* (No. 9) (data not shown). Transitions (A = G, T = C) and transversions (C = A) were observed at sites 344 and 548, and 30 and 147, respectively. The genetic distance values ranged from 0.0000 to 0.0048 and overall mean was 0.0017, which are also typical of intraspecific variation. Thus, based on mitochondrial and nuclear DNA data, the present study confirmed that *D. grandis* is a junior synonym of *D. balaenopterae* and thus, causative agent of human diplogonoporiasis should be called *D. balaenopterae*.

3.3. Taxonomic and phylogenetic relationships between the genera *Diplogonoporus* and *Diphyllobothrium*

The Pseudophyllidea van Beneden in Carus, 1863, a well-recognized order of tapeworms (Platyhelminthes: Eucestoda), has been considered to be a monophyletic group of difossate cestodes [40]. However, phylogenetic analysis based on nuclear ribosomal RNA genes and ITS-2 sequences revealed the presence of two phylogenetically unrelated groups, indicating paraphyly or polyphyly of the order Pseudophyllidea [15,17,19,20]; consequently, two new orders, Diphyllobothriidea and Bothriocephalidea, have been proposed to accommodate these unrelated lineages [41]. In addition another study [24], the mitochondrial DNA results of this study also support the proposal. However, the taxonomic and phylogenetic relationships between the genera *Diplogonoporus* and *Diphyllobothrium* (Diphyllobothriidae, Diphyllobothriidea) have been not yet been clarified in sufficient detail, primarily because DNA sequence data are only available for a limited number of species [42], and also because the taxonomic positions of several species within these genera are still uncertain.

Fig. 2 shows phylogenetic trees inferred by ML and BI algorithms using nucleotide sequences of the *cox1* (356 bp). Although two tree topologies were somewhat different, *Diplogonoporus* isolates formed monophyletic clade belonging to Diphyllobothriidae (Diphyllobothriidea) with strong nodal support (≥ 75 in ML, ≥ 0.97 in BI) and were more closely related to *D. stemmacephalum*, the type-species of the genus *Diphyllobothrium*, corroborating the findings of a previous study [24]. Phylogenetic studies on pseudophyllidean (= diphyllobothriidean and bothriocephalidean) cestodes support paraphyly and/or polyphyly of several cestode genera, including *Ligula* Bloch, 1782 and *Bothriocephalus* Rudolphi, 1808 [17,19,22], and *Diphyllobothrium* [43]. The close relationship between *Diplogonoporus* spp. and *D. stemmacephalum* implies that these taxa constitute a paraphyletic group.

Interestingly, *D. balaenopterae* and *D. stemmacephalum* are phylogenetically closely related and infect cetaceans, such as *Balaenoptera* spp. and *M. novaeangliae* (Balaenopteridae) [1,2], and the harbor porpoise (*Phocoena phocoena*, Phocoenidae, Cetacea), bottlenose dolphin (*Tursiops truncatus*, Delphinidae, Cetacea) and long-finned pilot whale (*Globicephala melas*, Delphinidae, Cetacea) [1], respectively. Based on these similarities in host preference, it seems likely that *D. balaenopterae* and *D. stemmacephalum* are derived from a common ancestral species [24].

The genus *Diplogonoporus* has been characterized as having two sets of genitalia in a single proglottid [1]. However, additional genitalia (3 to 5 pairs) have been observed in some segments of *D. balaenopterae* collected from an Antarctic sei whale (*B. borealis*, Balaenopteridae, Cetacea) [44]. Similarly, in dwarf forms of *D. balaenopterae* (body length: 61–809 mm, max. width 2.0–5.6 mm) obtained from a minke whale, although two sets of genitalia per segment were usually encountered, sometimes 4 paired genitalia were observed in a single proglottid in the same individuals [45]. Conversely, two sets of reproductive organs

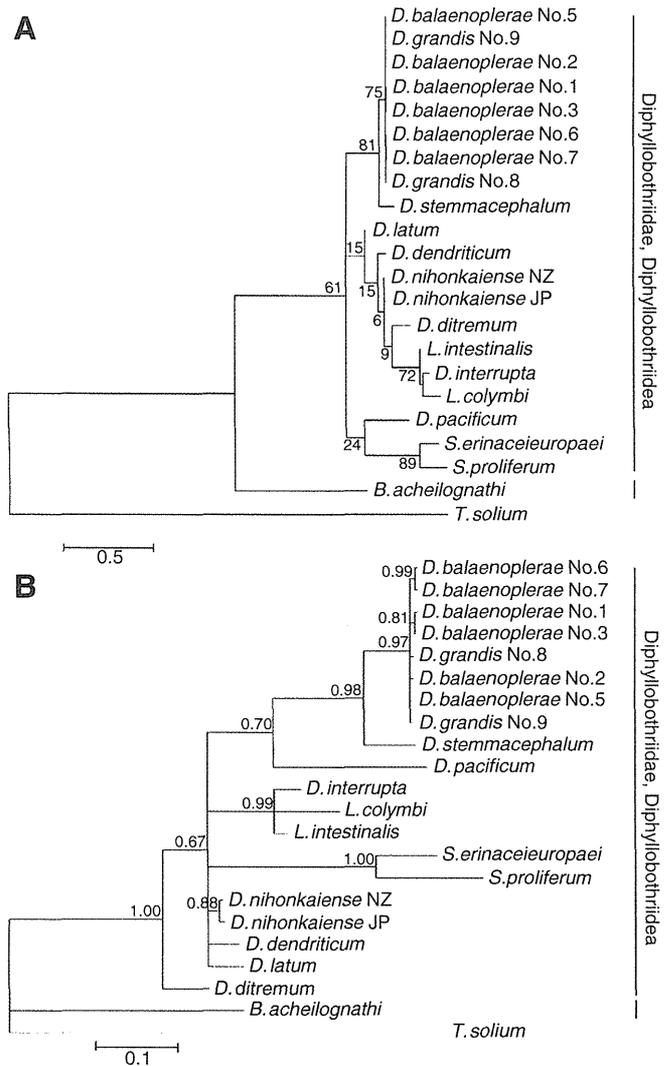


Fig. 2. Phylogenetic trees constructed by the (A) maximum likelihood and (B) Bayesian inference analyses using *cox1* sequence data (356 bp). Nucleotide sequence data were obtained from the following accession numbers: *Diplogonoporus balaenopterae* (AB355622–AB355624, AB355626, AB474567, AB474568), *Diplogonoporus grandis* (AB355628, AB355629), *Diphyllobothrium stemmacephalum* (DQ768191), *Diphyllobothrium pacificum* (AB517949), *Spirometra erinaceieuropaei* (AB369249), *Sparganum proliferum* (AB015753), *Digamma interrupta* (EU241311), *Ligula intestinalis* (EU241317), *Ligula colymbi* (EU241308), *Diphyllobothrium ditremum* (FM209182), *Diphyllobothrium latum* (AB510496), *Diphyllobothrium dendriticum* (AM412738), *Diphyllobothrium nihonkaiense* (AB268585, AB364645) and *Bothriocephalus acheilognathi* (Bothriocephalidae, Bothriocephalidea, HM439384). *Taenia solium* (Taeniidae, Cyclophyllidae, AB516957) was used as the outgroup. Bootstrap values (1000 replications) in ML and posterior probabilities in the BI are shown at the branch. Bars indicate the number of base substitutions/site.

per segment have been reported in *Diphyllobothrium yonagoense*, which infects Risso's dolphin (*Grampus griseus*, Delphinidae, Cetacea) [46] and rarely, duplicated genitalia have been reported in *Bothriocephalus*, *Triaenophorus*, *Echinophallus* and *Paraechinophalus* (Bothriocephalidea) [41]. Moreover, in some genera of Diphyllobothriidae (e.g., *Baylisia*, *Tetragonoporus* and *Hexagonoporus*), multiple genital organs have been observed in each segment. Thus, because the number of genitalia can vary, even within species, two sets of genitalia as a taxonomic character may not be sufficiently robust for resolving affiliations among the members of the genus *Diplogonoporus*. In addition, these observations suggest that multiplication of the reproductive organs may be an adaptive phenomenon in cestode evolution.

In so far as the taxonomic placement of *Diphyllobothrium pacificum*, a parasite of the South American sea lion (*Otaria flavescens*,

Otariidae, Carnivora), is concerned, it has been proposed that *Adenocephalus pacificus* Nybelin, 1931, the original name assigned to the species, should be restored as the valid name based on molecular and morphological data which *D. pacificum* is phylogenetically distant from *Diphyllobothrium* species (i.e., *D. nihonkaiense*, *D. latum*, and *D. dendriticum* etc) [23]. Our results also support the validity of the original genus *Adenocephalus* although the position of *D. pacificum* was different in two phylogenetic trees. Because *Diphyllobothrium* including *D. nihonkaiense*, *D. latum*, *D. dendriticum* and *D. ditremum*, which are all non-cetacean *Diphyllobothrium* parasites, is placed at markedly distant position from *D. stemmacephalum* (Fig. 2). The genera *Digramma* Cholodkovsky, 1914 and *Ligula*, formed a monophyletic cluster, but *Digramma* is considered a synonym of *Ligula* [22,47]. Indeed, *Digramma* may merely be a rare diplogonadic type of *Ligula*.

Regarding the validity of the genera *Diplogonoporus* and *Diphyllobothrium* based on molecular evidence, the following scenarios are possible;

- i) *Diplogonoporus* should be synonymized with *Diphyllobothrium*, and the name *Diphyllobothrium* should be used for *D. stemmacephalum*, which may also include several other cetacean *Diphyllobothrium* species
- ii) *Diplogonoporus* should be retained as a valid genus
- iii) Non-cetacean *Diphyllobothrium* species, including *D. nihonkaiense*, *D. latum* and *D. dendriticum*, should be placed in a newly designated genus.

To conclude whether *Diplogonoporus* can be regarded as a synonym of *Diphyllobothrium* or not, comprehensive molecular-phylogenetic analyses should be undertaken using other *Diplogonoporus* species, such as *Diplogonoporus tetrapterus*, which infects the harbor seal (*Phoca vitulina*, Phocidae, Carnivora) and fur seal (*Callorhinus ursinus*, Otariidae, Carnivora), and *Diplogonoporus violettae* which infects the sea lion (*Eumetopias jubatus*, Otariidae, Carnivora). In addition, the gigantic and dwarf forms of *D. balaenopterae* [45,46], as well as species that infect other cetacean species, including *D. yonagoense* and *Diphyllobothrium macroovatum* which infect minke whales (*B. acutorostrata*) and gray whale (*Eschrichtius gibbosus*, Eschrichtiidae, Cetacea) [48] should be also examined. Moreover, other genera related to *Diplogonoporus*, including *Tetragonoporus*, *Hexagonoporus* and *Polygonoporus*, are required in order to clarify the phylogenetic relationships among these cestodes, and molecular analyses using *D. yonagoense* and *Hexagonoporus* isolates are currently underway. For scenario iii), the genus *Diphyllobothrium* should contain the type-species of *D. stemmacephalum*. It may therefore be reasonable to place the non-cetacean *Diphyllobothrium* species into a new genus that is distinct from *Diphyllobothrium*. To revise the validity of the genus *Diphyllobothrium*, further molecular analysis using more non-cetacean *Diphyllobothrium* taxa would be necessary.

In conclusion, the findings of the mitochondrial and nuclear DNA analyses reported here will be very useful, not only for analyzing the phylogenetic relationships among eucestodes, but also for differentiating *Diplogonoporus* species from *Diphyllobothrium* species. In addition, larval stages of *D. balaenopterae* have not yet been discovered. It will now be possible to determine whether plerocercoids found in marine fish hosts are *D. balaenopterae* or not, which will further clarify the life cycle of this parasite and facilitate the prevention of diplogonoporiasis in humans.

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—面白い寄生虫の臨床(Ⅱ)—

日本獣医臨床寄生虫学研究会編

輸入寄生虫病 —マラリアと人獣共通寄生虫症を中心に考える—

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1 はじめに

—寄生虫症をめぐる法的変遷
と輸入感染症の位置づけ—

「感染症の予防及び感染症の患者に対する医療に関する法律」(以下、「感染症法」という.)は、従来の「伝染病予防法」「性病予防法」「エイズ予防法」の3つを統合して1998年に制定され、2007年には「結核予防法」も統合された。その間、SARS(重症急性呼吸器症候群)や新型インフルエンザといった地球規模の新興・再興感染症が問題となり、人権尊重を原則としつつ、緊急時における対策の強化としては、感染者に対する隔離といった措置も可能となる改正がなされた。また、SARSや鳥インフルエンザ以外でも、ウエストナイル熱、狂犬病といった人と動物の共通感染症(人獣共通感染症、動物由来感染症)の侵入と拡大を防止するため、医師のみならず動物の診療等に従事する獣医師にも、届出義務が新たに追加された。獣医師の届出対象となる罹患動物としては、SARSや出血熱といった重篤なウイルス性疾患を想定したサルやプレーリードック、ハクビシン、タヌキなどのほか、鳥インフルエンザやウエストナイル熱で「鳥類に属する動物」、エキノコックス症で「犬」が追加されるに至っている。

寄生虫症対策の指針だった寄生虫病予防法(1931年制定)は、国内感染状況の変化を背景に、感染症法の制定前の1994年に廃止された。寄生虫予防法で主に対象とされたのは、回虫、鉤虫、住血吸虫、肝吸虫であったが、感染症法で、人症例の届出が義務づけられている寄生虫症は、マラリアとエキノコックス症(以上、第4類)、赤痢アメーバ、ジアルジア症、クリプトスポリジウム症(以上、第5類)である。対策の重点とされる寄生虫症がこの間大きく変わったことがよくわかるが、回虫や鉤虫が宿主特異性の強い寄生蠕虫で、専ら人の感染

症としてのみ問題となっていたのに対し、感染症法での届出対象寄生虫症は、全て人獣共通感染症と言ってよい。

このうち、マラリアは、2003年の検疫法改正で、検疫感染症としても位置づけられた。従来、代表的な検疫感染症としては、国際衛生規則(1951年)で定められたペスト・コレラ・黄熱病をあげるのが常であったが、前述の感染症法の制定(1998年)にあわせ、まずペスト以外の第1類感染症(エボラ出血熱、マールブルク熱、ラッサ熱など)が追加された。さらに、SARSの発生と拡大(2003年)の後には、鳥インフルエンザや新たに指定された第1類感染症(SARS、痘そう)、と並んで、第4類感染症からマラリア・デング熱が追加されるに至った。日本国内でも *Vibrio* 属細菌の常在が確認されるコレラや、日本国内にベクターが生息していない黄熱病が検疫対象からぬけ、日本国内にベクターが生息しているデング熱やマラリアが検疫対象となったのは、一貫した考えに基づく理にかなった改正と言える。チクングニア熱ウイルスのベクターは、デング熱ウイルスと共通の種の蚊が多いが、ウイルスの伝播効率が非常に高いこともあって、年間の患者報告数が10例にみえない段階で、2011年に速やかに4類感染症に分類されるとともに、検疫対象感染症にもなった。一方、人獣共通寄生虫症の中には、感染症法での指定を受けていなくとも、第1類感染症に匹敵するような致死率を有するものや、感染動物の移入が、ベクターを介した国内での新興・再興感染症のリスクに直結するものもある。

2 問題となる輸入寄生虫病

—人獣共通寄生虫症(動物由来寄生虫症)を中心に—

ある感染症が、実際に日本国内で新興・再興感染症となる可能性については、生態・環境変化以外にも多くのファクターが関わり、病原体の種ごとに別々に考えねばならない。以下に代表的なものを列挙し、最近の動向を示したい。

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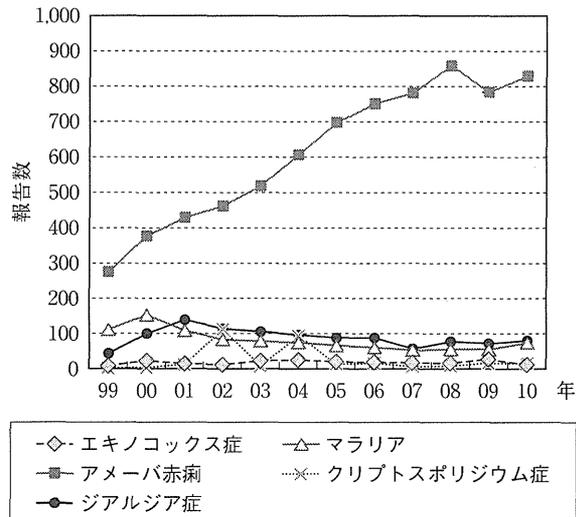
(1) マラリア

人に寄生するマラリア原虫としては、従来熱帯熱マラリア原虫 *Plasmodium falciparum*、四日熱マラリア原虫 *P. malariae*、三日熱マラリア原虫 *P. vivax*、卵型マラリア原虫 *P. ovale* の4種が知られ、サルに寄生するマラリア原虫のうち何種類かは特殊な環境下で人にも寄生するものの、公衆衛生的な問題は起こさないとされてきた [1]。日本での輸入感染症としてのマラリアを考えると、報告数は2000年までは増加傾向を示したが、2000年の年間154例をピークとした後は減少傾向を示し、最近では50～60例と横ばいである (図1)。その理由としては、マラリア対策の強化により世界的にマラリア患者数が減少していることもあげられる。特に、日本からの渡航者が多いアジア、オセアニア (南太平洋地域) では、近年マラリア対策が進展し、罹病率や死亡率が減少するとともに、流行の中心が熱帯熱マラリアから三日熱マラリアへとシフトした [2]。しかし、アジア地域では朝鮮半島や中国の一部で、温帯地方における再興感染症として三日熱マラリアが流行するとともに、東南アジアでは新興感染症として、*P. knowlesi* 感染が問題となっている。

P. knowlesi は、従来、マカク属のサルに感染するマラリア原虫として知られていたが、人での感染例も集団でみられるなど、従来のサル由来のマラリア原虫での人感染例とは異なった様相を示し、東南アジア諸国では第5の人マラリアとして脅威となっている [3]。*P. knowlesi* は、マカク属サルに寄生するマラリア原虫だが、24～28時間という早い赤血球内増殖期間もあって、原虫密度が高くなって重症化し、致死的となることもある。複数の抗マラリア薬を投与したのにも関わらず死亡した例では、薬剤耐性よりも、急速な経過が主因の可能性が高い。また、日本国内でも旅行者で発症例が報告されており、今後も輸入感染症として問題となる可能性がある。ただし、ベクターとなるハマダラカを介しての人から人への感染は、まだ確認されておらず、サル→蚊→ヒトでの濃厚接触がみられる地域でないと、クラスターでの人感染例はないと思われる。また、媒介蚊は、東南アジアの森林に生息する *Leucosphyrus* グループのハマダラカに限られ、日本国内に生息するハマダラカには伝播能力はない。

(2) 肉胞子虫

馬肉の生食に起因すると思われる食中毒の病原体として、近年、肉胞子虫類の *Sarcocystis fayeri* が注目されている。平成14年頃から、生食後5時間程度で、一過性の下痢と嘔吐を示すが (24時間以内にほぼ消失)、原因食品がわからない例が、報告されるようになり、食中毒にならない有症苦情例が増加する傾向を示した。原因の究明が進められた結果、平成23年4月25日、厚生労働省薬事・食品衛生審議会食品衛生分科会、食中毒・乳肉水産食品合同部会において、*Kudoa septempunctata* と *S. fayeri* が食中毒病因物質と認定された。さらに、厚労省通知 (6月17日付け) として、ヒラメ (*K. septempunctata*) 及び馬肉 (*S. fayeri*) の摂取に関連する食中毒リスク低減を図るため、生食用には-20℃での冷凍処理 (4時間以上—クドア、48時間以上—ザルコシスチス) を義務づけるに至った [4]。



届出対象寄生虫症は、全て人獣共通感染症と言ってよい。アメーバ赤痢が最も多い。マラリアは年間100例前後で推移していきながら、やや減少傾向にある。クリプトスポリジウム症は、水系集団感染があった年には多くなる。

図1 感染症法対象の寄生虫疾患年次報告数の推移

動省薬事・食品衛生審議会食品衛生分科会、食中毒・乳肉水産食品合同部会において、*Kudoa septempunctata* と *S. fayeri* が食中毒病因物質と認定された。さらに、厚労省通知 (6月17日付け) として、ヒラメ (*K. septempunctata*) 及び馬肉 (*S. fayeri*) の摂取に関連する食中毒リスク低減を図るため、生食用には-20℃での冷凍処理 (4時間以上—クドア、48時間以上—ザルコシスチス) を義務づけるに至った [4]。

馬肉生食によるザルコシスチス食中毒が、なぜこの時期に問題になったかについては、色々な理由が考えられるが、原料となる馬肉の生産地の変化も可能性の一つとして考えることができる。寄生例は、国産馬肉検体に比して、輸入馬肉検体で多く確認されているが、ザルコシスチス食中毒が問題となり始めた頃に、日本に食肉を目的として輸入される馬の原産国は、大きく変化している (図2)。

(3) 住血吸虫症とエキノкокクス症

日本住血吸虫症は、1977年以降、国内感染例は報告されていないが、ベクターであるミヤイリガイ *Oncomelania hupensis nosophora* は、未だ甲府盆地や千葉県の小櫃川流域に生息している (図3)。フィリピンや中国で得られる日本住血吸虫の株は、日本のベクター *O. h. nosophora* にも感染性を有している [5]。また、日本住血吸虫は、人に感染する住血吸虫の中では、一番保有宿主となる動物の数が多く、馬や牛、豚や犬・猫にとどまらず、ウサギやネズミなど多くの哺乳類が終宿主になる。食肉用の家畜動物や実験動物だけではなく、ペットとして輸入される動物による日本国内への持ち込み

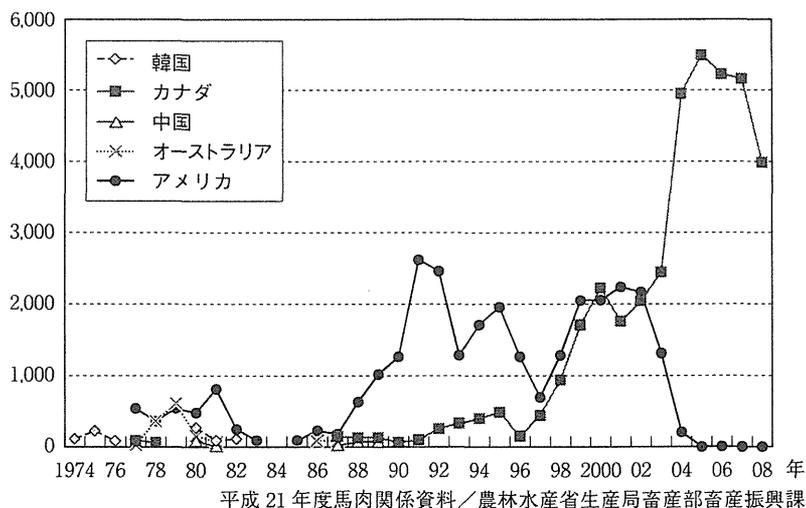


図2 食肉を目的とした馬の輸入頭数と原産国の推移

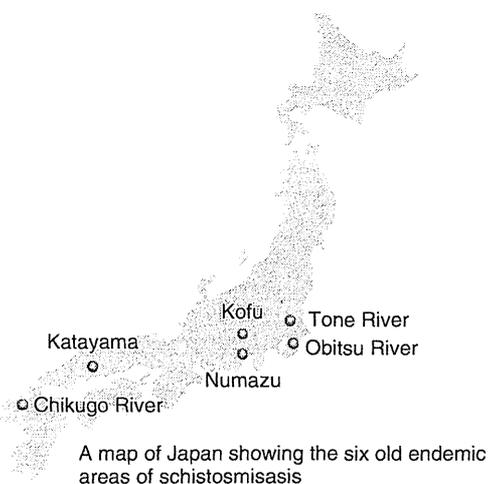


図3 日本国内における日本住血吸虫症の旧流行地と現在の媒介貝生息状況

	最後の虫卵陽性者の確認	媒介貝の生息確認
筑後川流域 (福岡・佐賀県)	1975	1983
片山地方 (広島県)	1967	1973
甲府盆地 (山梨県)	1977	現存 (1,300ha)
沼津地方 (静岡県)	1962	1961
小櫃川流域 (千葉県)	不詳	現存 (10ha)
利根川流域 (千葉・茨城県)	1973	1973

可能性も否定することはできない。

エキノコックス症では、単包条虫または多包条虫の幼虫感染が問題となる。キタキツネと同様、犬が感染したエゾヤチネズミを捕食すると、約1カ月後小腸内で成虫になり、感染源となり得るので、前述のように獣医師による届出も必要とされるようになった。最近、好適宿主ではない馬でも幼虫寄生が問題となることが報告された[6]。馬での多包条虫の幼虫寄生で生じる病変は、病理組織学的には肝砂粒症で、「表面に境界明瞭な粟粒大で、球形の硬い白色結節が散発している。結節は断面にも散在性に認められた。馬円虫や無歯円虫の幼虫による病変。過去においては日本住血吸虫の寄生でこのような病変が生じた。」(食肉・食鳥衛生検査マクロ病理学カラーアトラス 165頁 —全国食肉衛生検査所協議会編— 1997)とされる。日本住血吸虫症の軽症例(人)や非好適終宿主での病変でみられる病理組織所見で、超音波検査やX線検査などの画像検査での所見も類似すると思われる。エ

キノコックス症では、従来から犬の国内移動に伴う感染拡大のリスクが指摘されてきた。輸入寄生虫症ではないが、今後は、国内での動物の移動に伴うエキノコックス症の感染拡大について、従来とは異なった視点からの注意が必要とされるかもしれない。

(4) アライグマ回虫

米国においては1981年の初発例以来、アライグマ回虫 *Baylisascaris procyonis* の感染を原因とする重症脳障害患者が少なくとも10例以上確認され、そのために少なくとも5人が死亡している(表1)。日本国内での、人感染事例は、幸い現在まで報告されていない。しかしながら、動物園及び観光施設で飼育されているアライグマには本虫の寄生が見つかっており、東日本の観光施設では、2000年にウサギの群れで、アライグマ回虫による脳幼虫移行症が、クラスターとして確認された。アライグマが検疫対象となった2000年以降、日本国内への輸入は事実上困難になったが、1977年のアライグマを

表1 アライグマ回虫による人体幼虫移行症（米国での
主な報告例）

年齢	性別	患者居住地	診断	転帰	診断根拠	年次
10カ月	男	ペンシルバニア	脳炎	死亡	虫体(剖検)	1980
18カ月	男	イリノイ	脳炎	死亡	虫体(剖検)	1984
13カ月	男	ニューヨーク	脳炎	失明・発育不全・麻痺	血清	1990
13カ月	男	カリフォルニア	脳炎(網膜炎)	後遺症・発育不全	虫体(生検)	1993
29歳	男	カリフォルニア	網膜炎	治癒	虫体(検眼)	1993
13カ月	男	ミネソタ	脳炎	死亡	血清	1996
19カ月	男	ミネソタ	脳炎	死亡	血清	1997
11カ月	男	カリフォルニア	脳炎	後遺症・発育不全	血清	1998
2.5歳	男	イリノイ	脳炎	後遺症	血清	2000
17歳	男	カリフォルニア	脳炎	死亡	虫体(生検)	2000
11カ月	男	カリフォルニア	脳炎	後遺症・失明・痲癩	血清	2002

(Baylisascariasis. Gavin PJ, Kazacos KR, Shulman ST, Clin Microbiol Rev, 18 (4) : 703-18 (2005) ほか)

主人公としたテレビアニメ放映によるブーム以来、多くのアライグマが輸入され、それに伴いアライグマ回虫も日本国内に流入したと推測される [7-9].

日本国内の諸施設や一般家庭で飼育されたアライグマは現在までに総計2万頭を越えると推計されるが、その一部が飼育しきれずに逃亡や遺棄されたため、野外で定着・繁殖している現状が問題視されている。今までの調査では、これらの「野生アライグマ」からはアライグマ回虫の寄生例は確認されていないが、調査自体が散発的なものにとどまり、組織的調査やモニタリングは、必要性が叫ばれながらも実現されいない。また、2011年には、伴侶動物のキンカजू（アライグマ科）で、アライグマ回虫感染が疑われたが、*Baylisascaris* 属線虫の帰属については議論があり、キンカजूで検出される回虫とアライグマから検出される回虫についても、異同ははっきりしない。ところで、アライグマ回虫の人での幼虫移行症の致死率は、一類感染症に分類されるウイルス性出血熱とほぼ同じ水準で（表2）、非常に重篤な感染症と言ってもよいが、人-人感染が問題となることはないためか、感染症法や検疫法では、全くとりあげられていない。

3 検疫による動物由来感染症防御の考え方

現在、動物検疫については、感染症法や検疫法以外に、家畜伝染病予防法や狂犬病予防法、水産資源保護法

表2 主な一類感染症とアライグマ回虫幼虫移行症の比較

	報告数 流行地 (年次)	輸入感染者・ 報告数 先進国 (年次)	報告数・ 日本 (年次)	致死率 (%)
エボラ出血熱	1,617 アフリカ中央部 (1976~2002)	なし	なし	50~80
マールブルク熱	500以上 アフリカ中東南部 (1975~2005)	38 ドイツなど (1967~2008)	なし	30~70
ラッサ熱	20~30万人? アフリカ西部 (1969~)	23+ ドイツ, 米国等 (1969~)	1 (1987)	1~2
クリミア・コンゴ出血熱	数十名 アフリカ, 中央アジア等	不明	なし	15~40
アライグマ回虫脳炎	不明 米国, ヨーロッパ諸国以外	20+ 米国, カナダ, ドイツ (1980~2012)	なし	50程度

アライグマ回虫 *Baylisascaris procyonis* による脳炎では、致死率は50%近く、重篤な後遺症なく治癒することはほとんどない。

(感染症の話 エボラ出血熱 佐多徹太郎 2002年第32週号, ラッサ熱 倉田 毅 2002年第35週号, マールブルク熱 倉田 毅 2002年第36週号, クリミアコンゴ出血熱 倉田 毅 2002年第31週号, IDWR 国立感染症研究所 ほか)

表3 主な検疫対象動物の係留期間

動物種類	係留期間	
	輸入	輸出
偶蹄類の動物	15日	7日
馬	10日	5日
鶏, ウズラ, タチョウ, 七面鳥, かも目の鳥類	10日	2日
初生ひな	14日	2日
上記以外	1日	1日

による検疫が行われているが、準拠する法律で、主たる検疫目的は異なっている。現在の検疫検査システムでは、狂犬病やウイルス性出血熱など特定の病原体検査を除くと、係留期間内に発症しないことの確認が、生体の動物検疫の中で、大きな役割を占めていると思われる。しかし、人でも他の動物でも、発症までの期間が月単位のことが多い寄生虫症の場合、定められた係留期間内に明らかな病状を示す可能性は高くない（表3）。

検疫の基本は、国外・地域外からの危険な感染症の侵入・蔓延の防止という社会防衛の考え方に立っている。マラリア以外にも、国内発生がなく（あるいはほとんどなく）、国内にベクターや保有動物が生息している寄生虫症は複数ある。現在、獣医師に届出義務のある主な感

染症罹患動物は、サル（エボラ出血熱、マールブルク熱、細菌性赤痢、結核）、イヌ（エキノコックス症）、プレーリードック（ペスト）、鳥類（鳥インフルエンザ-H5N1、ウエストナイル熱）、ハクビシンやタヌキなど（SARS）である。また、「動物展示施設における共通感染症対策ガイドライン（2003）」では、オウム病クラミジア、アライグマ回虫、ウエストナイル熱ウイルス等に、注意が払われている。個々の感染症で、各々実際にあった防疫を考える場合は、症状の強さや致死率、感染力の違いや想定されるリスクなど、総合的に考えていかねばならない。人獣共通感染症については、人・動物の両面から検疫対象疾患が議論されてきたが、寄生虫感染症については、ウイルスや細菌に比して、十分な検討がなされてこなかった印象を受ける。今後、代表的な家畜・伴侶動物によって輸入される可能性がある寄生虫症について、情報を収集・共有しながら、防疫を具体化していく必要があると思われる。

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講座

食品による寄生動物感染症¹

食品媒介寄生虫感染症

— 総論・旅行医学における本症 —

大前 比呂思

〈掲載予定内容、所属・著者、掲載巻号〉

- 1. 食品媒介寄生虫感染症
(寄生虫学的総論・旅行医学の中での本症)
国立感染症研究所 大前比呂思 (40-10)
- 2. 食品媒介寄生虫食中毒の実態と対策—行政の立場から—
厚生労働省 温泉川肇彦 (40-10)
- 3. 原虫感染症(1)サルコシスティス・クドア
国立感染症研究所 八木田健司
- 4. 原虫感染症(2)クリプトスポリジウム・ジアルジア
国立感染症研究所 泉山信司
- 5. 原虫感染症(3)トキソプラズマ
国立感染症研究所 永宗喜三郎
- 6. 蠕虫感染症(1)横川吸虫・肝吸虫
国立感染症研究所 森嶋康之
- 7. 蠕虫感染症(2)肺吸虫
国立感染症研究所 杉山 広
- 8. 蠕虫感染症(3)糸虫
国立感染症研究所 山崎 浩
- 9. 蠕虫感染症(4)アニサキス
麻布大学 川上 泰・国立感染症研究所 梅原梓里
- 10. 蠕虫感染症(5)トキソカラ (犬回虫・猫回虫)
麻布大学 平 健介
- 11. 蠕虫感染症(6)顎口虫
宮崎大学 野中成晃
- 12. 蠕虫感染症(7)旋毛虫
国立感染症研究所 川中正憲

はじめに

日本国内でも、かつて回虫や鉤虫といった消化管の寄生蠕虫症が全国的に蔓延していた。また、地域によっては、住血吸虫や肝吸虫、肺吸虫やフィラリアなどの寄生虫症も多く見られた。現在、日本国内での日常診療の場で、これらの寄生虫症にふれることは少なくなったが、熱帯・亜熱帯の途上国では、未だに大きな公衆衛生上の問題である。近年、これらの寄生虫症は、感染者数が多いものの種々の理由から対策が遅れがちだった一連の感染症、Neglected Tropical Disease (NTDs) として、世界保健機構 (WHO) を中心に国際機関の注目を集めており、昆虫媒介性のリンパ系フィラリア症は、2020年までの世界的な制圧を目指している。しかし、回虫や鞭虫、赤痢アメーバやジアルジアといった、飲料水や食物から容易に感染する消化管の寄生虫は、世界的にみると現在も、全体で何十億という感染者がいると推定されている¹⁾。

1. 寄生虫とは

寄生虫とは、宿主と言われる他の生物に寄生し栄養を受ける生物のうち、動物に分類されるものを総称する言葉で、寄生動物と言われることもある。寄生部位によって、体表面に寄生するものを外部寄生虫、体内に寄生するものを内部寄生虫と

分類するが、一般に寄生虫と言った場合は、主に内部寄生虫のことを指す。寄生虫は、多くの動物種にまたがっているが、厳密な生物学的分類による各寄生虫種の帰属については、曖昧な点や異論も多く、医学的な観点からみるとあまり実用的な意味はない。慣用的には、そのライフサイクル（生活環、生活史）の中で、他の動物への寄生生活の時期を持つ原生動物を原虫 protozoa と総称し、多細胞の後正動物を蠕虫 helminths と総称する。原虫は、無性生殖しか行わない根足虫類や鞭毛虫類、有性生殖も行う孢子虫類などに大別される。また、蠕虫は、袋形動物に属して雌雄異体の線虫 nematodes と、扁平動物に属し原則として雌雄同体の吸虫 trematodes、条虫 cestodes などに大別される。このように、寄生虫と総称される動物は、赤血球に寄生し体長1~2 μm のマラリア原虫から、消化管に寄生し体調10m 近くにも及ぶ広節裂頭条虫まで、実に多彩な動物種の集合体である。

寄生生活に適應すると、多くの種では、自由生活に必要な器官を失っていく。消化管の蠕動運動に発達した筋層で対抗する回虫のような例を除き、運動器官は退化していくのが一般的である。消化器官は、退化もしくは独自の発達を示し、吸虫では盲管に終わり、条虫では固有の消化器官を欠くものの上皮全体から栄養分を吸収できるようになっている。生殖器官についてはよく発達していることが多い。

寄生虫の宿主となる動物も多岐にわたるが、次世代につながるように原虫が無性・有性生殖を行い、蠕虫の成虫が寄生する宿主を終宿主 Final host と呼ぶ。また、有性生殖のステージを持つ原虫も無性生殖のみを行い、蠕虫の幼虫が寄生して体内で増殖する宿主は、中間宿主 Intermediate host である。一般的には、終宿主内では寄生生活としての適應がうまく進み、寄生虫は宿主に病害性を示しながらも、長期間にわたって共生を続ける場合が多い。一方、中間宿主は、変異・増殖の場として、寄生虫が利用している動物と位置づけることもでき、宿主の生命を脅かすような病害性を示すことも稀ではない。また、生活環の中で不可欠な中間宿主ではないが、寄生虫が侵入

後も変異せずに、生存可能な宿主を待機宿主と呼ぶ。ヒトの位置づけについては、寄生虫の種によって異なっており、終宿主として位置づけられるものもあれば、中間宿主や待機宿主として位置づけられる場合もある。終宿主となり得る動物種の数は、寄生虫の種によって異なり、総じて吸虫や条虫では多い。

大半の寄生虫では、その生活環の中に他の動物も関係しており、人獣共通感染症（動物由来感染症）zoonosis の病原体となるものも多い。ヒトが終宿主となる場合、ヒト以外の終宿主動物を保虫宿主 Reservoir host と呼ぶ。病害性は、寄生部位によっても様々だが、ヒトを本来の固有宿主としない蠕虫の幼虫が感染すると、成虫になることができず体内を移動するうえ、有鉤囊虫症やエキノコックス症のように、種によっては無性生殖を繰り返し、たいへん重篤な症状を引き起こすことがある（幼虫移行症 Larva migrans）。また、通常は無害で不顕性感染している寄生虫が、後天性免疫不全や抗がん剤・免疫抑制剤投与などを契機として、虫体の増殖と病態の顕在化・重症化を起こす場合、日和見感染症 Opportunistic infection と呼ばれる。

2. 食中毒との関連

寄生虫は、寄生生活から寄生生活、自由生活から寄生生活へと、その生活環の中で、生活環境を変えるが、その過程の中で、環境中からヒトへ、ヒトからヒト、他の動物からヒトへと移動することになる。その移動過程は、マラリア原虫のように蚊によって媒介されることもあるが、媒介動物（ベクター vector）の介在なしに直接経口的、経皮的になされることも多い。経口的に感染する寄生虫の感染型が、食物中、或いは、飲料水中に存在する場合、Foodborne disease, Waterborne disease を起こし、食中毒の原因として特定されることになる。寄生虫は、その生活環の中で様々なその姿を変え、同種の寄生虫であっても、ヒトへの感染性を持つ時期は、その生活環の中で限られている。この点、同じように食中毒を起こす他の病原体、ウイルスや細菌とは大きく異なる。