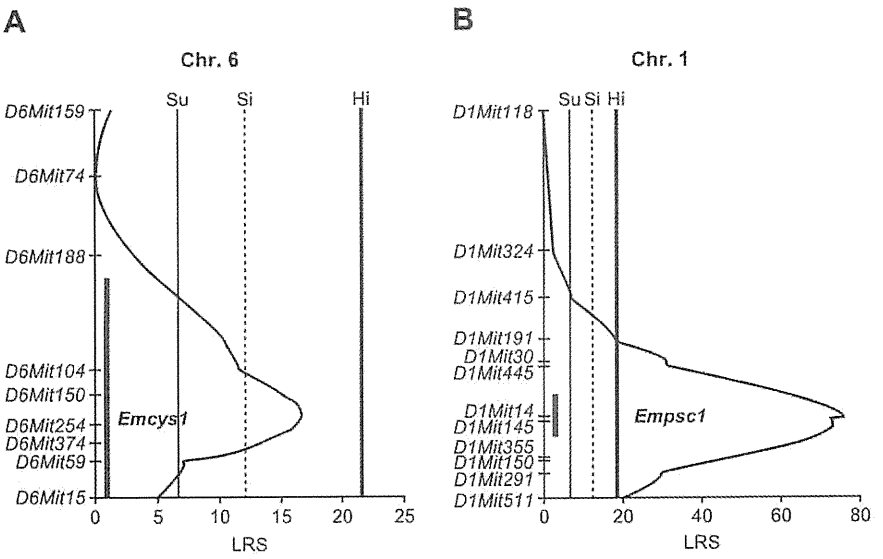

Fig. 3. Quantitative trait loci (QTLs) affecting cyst establishment (A) and protoscolex development (B) of *Echinococcus multilocularis* in the liver. The QTLs on chromosomes (Chrs.) 6 (*Emcys1*) and 1 (*Empsc1*) showed significant and highly significant linkage to (A) cyst establishment and (B) protoscolex development, respectively. The gray bars on the graph indicate approximate 95% confidence intervals. The thin, dotted and thick lines represent suggestive (Su), significant (Si) and highly significant (Hi) thresholds calculated by 1,000 times permutation tests, respectively. The microsatellite markers used for determining genotypes of N_2 mice are presented along the Y-axis. LRS, likelihood ratio statistic.

Table 2
Quantitative trait loci (QTLs) with high likelihood ratio statistic (LRS) scores (>4.0) detected by marker regression analysis.

Chr.	Locus	LRS	Contribution (%)	*P* value	CI	Additive effect
Cyst establishment						
3	*D3Mit164*	4.8	4	0.02847	113	−14.07
6	*D6Mit104*	13.1	10	0.00030	43	−22.72
6	*D6Mit150*	15.0	12	0.00011	38	−24.32
6	*D6Mit254*	14.7	12	0.00013	38	−24.05
6	*D6Mit374*	11.0	9	0.00092	51	−20.95
6	*D6Mit59*	7.2	6	0.00724	76	−17.14
6	*D6Mit15*	4.3	4	0.03725	124	−13.37
9	*D9Mit91*	6.7	5	0.00989	82	16.44
13	*D13Mit9*	5.7	5	0.01724	96	−15.21
13	*D13Mit148*	5.3	4	0.02152	103	−14.73
13	*D13Mit262*	5.2	4	0.02211	103	−14.63
17	*D17Mit187*	4.5	4	0.03457	121	13.55
17	*D17Mit221*	4.0	3	0.04627	136	12.78
Protoscolex development						
1	*D1Mit415*	7.2	6	0.00734	76	−0.75
1	*D1Mit191*	17.9	15	0.00002	32	−1.15
1	*D1Mit30*	31.4	24	0.00000	19	−1.48
1	*D1Mit445*	31.4	24	0.00000	19	−1.48
1	*D1Mit14*	75.4	48	0.00000	10	−2.12
1	*D1Mit145*	73.5	48	0.00000	10	−2.09
1	*D1Mit355*	42.5	31	0.00000	15	−1.70
1	*D1Mit150*	40.4	30	0.00000	16	−1.68
1	*D1Mit291*	29.5	23	0.00000	20	−1.46
1	*D1Mit511*	18.5	15	0.00002	31	−1.18
2	*D2Mit296*	4.2	4	0.03958	127	−0.58
3	*D3Mit182*	4.4	4	0.03551	122	0.59
5	*D5Mit222*	5.4	5	0.02063	101	−0.67
6	*D6Mit188*	7.1	6	0.00765	77	0.74
14	*D14Mit165*	6.2	5	0.01274	88	−0.71
17	*D17Mit198*	4.5	4	0.03307	119	0.60
17	*D17Mit139*	7.3	6	0.00679	75	0.76
17	*D17Mit89*	5.6	5	0.01809	97	0.66
17	*D17Mit218*	9.2	8	0.00247	60	0.85
17	*D17Mit187*	10.3	9	0.00130	54	0.89
19	*D19Mit69*	6.0	5	0.01397	90	−0.69
X	*DXMit186*	5.9	5	0.01557	93	0.70

Chr., chromosome; CI, confidence interval.

D2 and DD and between F_1 and DB (Fig. 4B). A list of *Empsc1* candidates on Chr. 1 is shown in Table 3.

4. Discussion

As shown in a previous investigation, susceptibility/resistance to *E. multilocularis* infection was different among mouse strains including D2 and B6 mice (Matsumoto et al., 2010). D2 mice were more susceptible to infection than were B6 mice. D2 mice showed a higher number of cysts established in the liver after administration of infective eggs than those of B6, and at 16 weeks p.i., maturation of protoscoleces occurred only in D2, but not in B6 mice. The objective of this study was to perform a genome-wide linkage analysis of a cross between D2 and B6 mice to localize genes associated with the increased susceptibility of D2 mice against *E. multilocularis* infection.

We found significant linkage between cyst establishment and genotypes at *Emcys1* on Chr. 6 with a peak LRS score of 15.0, accounting for 12% of the variation (Table 2). The segregation analysis supported a strong influence of *Emcys1* on cyst establishment (Fig. 4A). However, the continuous distribution of N_2 phenotypes implies that multiple genes control susceptibility to *E. multilocularis* infection. Although statistical modeling of the current data did not detect any QTL interacting with *Emcys1*, the suggestive QTL on Chr. 9, and even others slightly below the suggestive level (LRS = 6.7) such as those on Chrs. 3, 13 and 17 (Fig. 2A), may have an influence on susceptibility. At this time, it is difficult to identify a gene responsible for cyst establishment on *Emcys1*, a region between *D6Mit188* and *D6Mit15*, as there are nearly 1,000 genes located within this chromosomal region. Even with the aid of the web-based program Positional Medline (PosMed; http://omicspace.riken.jp/PosMed) (Yoshida et al., 2009), approximately 200 immune-related genes that may be involved in host–parasite interplay, were identified, some of which are listed in Table 3.

The safety requirements for handling infective *E. multilocularis* eggs hinder the research on cyst establishment after oral infection with eggs and thus only limited information is available for further discussion on candidate genes. Gottstein et al. (2010) used

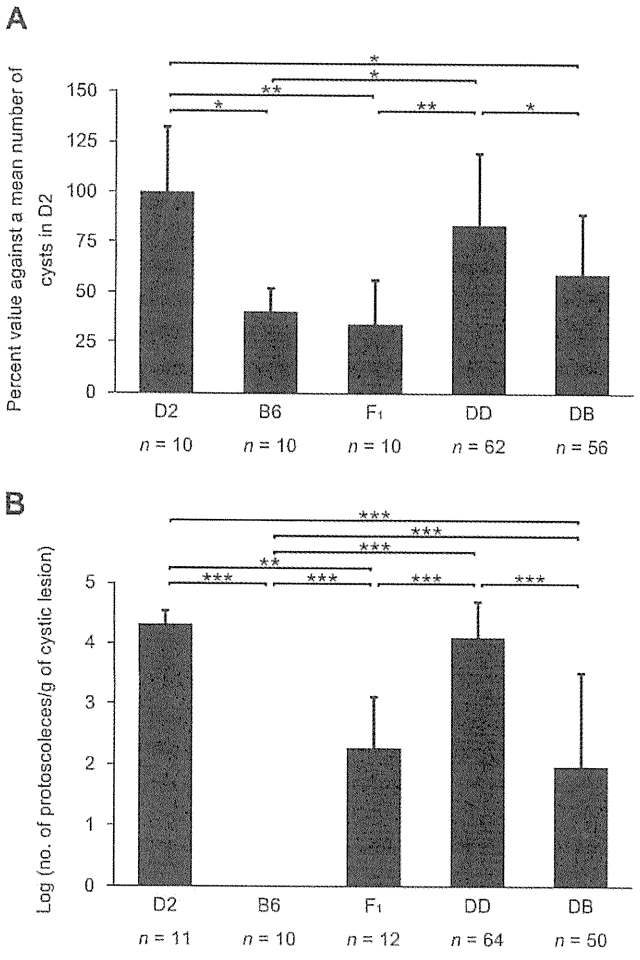

Fig. 4. Effect of allelic combination at *D6Mit150* (A) and *D1Mit14* (B) on N_2 mice. N_2 mice were segregated according to their respective genotypes at (A) *D6Mit150*, a marker representing the *Emcys1* locus and (B) *D1Mit14*, a marker representing the *Empsc1* locus. The mean value of each group was shown with the bar representing the S.D. DD and DB indicate individuals homozygous for the D2 allele and heterozygous for D2 and B6 alleles, respectively. The number of animals in each group is indicated at the bottom. Statistical analysis for multiple comparisons between the mean values of each group was conducted using Scheffé's *F* test (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$).

microarray analysis to compare the expression levels of hepatic genes in mice between pre- and 31-days p.i. of eggs. The study identified only 38 genes whose expression levels were significantly changed. None of those were located on *Emcys1*, implying that the host factors, apart from those expressed in the liver, may have more influence on the cyst establishment and that the earlier stages of infection such as hatching, activation, penetration, migration and predilection of oncospheres might be determinants of susceptibility to the infection. As recently reviewed by Vuitton and Gottstein (2010), the cell-mediated immune responses, especially acute inflammatory Th1 response, are known to play an important role in the early stage of *E. multilocularis* infection. Furthermore, early inflammation induced by complement activation was shown to be important in controlling the establishment of the metacestode of *Echinococcus granulosus*, a closely related parasite (Breijo et al., 2008). The *Emcys1* locus includes genes related with the Th1 response, such as *Cd4*, *Lag3* and *Cd69*, and those associated with complement components, such as *C1s*, *C1r*, *C1rl* and *C3ar1* (Table 3).

In the Chinese village of Nanwan, a highly endemic area for AE, only certain family members were likely to be more susceptible to AE than others, despite sharing similar life patterns (Yang et al., 2006), implying that host genetic factors contribute to the susceptibility to *E. multilocularis* infection in humans. Some genes showing quantitative genetic variation in mice have also been revealed to affect the phenotypic variance in humans (Korstanje et al., 2004; Hillebrandt et al., 2005; Peters et al., 2007). Therefore, the data obtained in this study could provide an experimental basis for further identification of human genetic factors that are associated with the response after ingestion of *E. multilocularis* eggs. Such information is expected to facilitate the development of effective disease prevention and control programs, for example, an intensive screening of genetically high-risk groups for AE infection.

The identification of genetic factors affecting the protoscolex development of *E. multilocularis* will lead to a better understanding of host and parasite interplay in the intermediate hosts. In our analysis, highly significant linkage was observed between protoscolex development and genotypes at *Empsc1* on Chr. 1 (a peak LRS score of 75.4) (Fig. 3B). A significant influence of *Empsc1* on variants was demonstrated by a high value of contribution (48%) (Table 2) as well as significant differences between DD homozygous and DB heterozygous in segregation analysis (Fig. 4B), whereas the detection of other suggestive QTLs suggests that the protoscolex development is under multigenic control, as in the case of cyst establishment.

The physical barrier between growing parasites and host tissue is the laminated layer, an acellular and carbohydrate-rich outer membrane of cysts, which protect parasites from direct contact with host immune cells. Meanwhile, several host-derived molecules were previously reported to exist in the cyst fluid or on the wall (Kassis and Tanner, 1977; Ali-Khan and Siboo, 1981), indicating that certain host-derived factors are transported through the laminated layer and are involved in parasite development in the hosts. In recent years, great progress has been made in elucidating the mechanisms of parasite development in the hosts by identifying the evolutionarily conserved signaling systems in *E. multilocularis*, which can interact with host-derived molecules (Konrad et al., 2003; Spiliotis et al., 2003, 2005, 2006; Zavala-Góngora et al., 2003, 2006, 2008; Gelmedin et al., 2008, 2010; Brehm, 2010; Förster et al., 2011). Specifically, interactions between parasite receptors and host-derived molecules, such as epidermal growth factor and insulin have been demonstrated experimentally (Spiliotis et al., 2006; Gelmedin et al., 2008; Brehm, 2010), strongly suggesting that parasite development is triggered by host signaling systems. Of 450 genes located on the *Empsc1* locus, more than 100 were previously associated with various kinds of signaling systems in mammals, as exemplified in Table 3. Thus, a better understanding of evolutionarily conserved *E. multilocularis* signaling systems may lead to further identification of host factors important for parasite development from the existing list of candidates.

In humans, major histocompatibility complex (MHC) genotypes were associated with the clinical severity of AE (Eiermann et al., 1998; Godot et al., 2000; Zhang et al., 2003). In mice, the genes coding for MHC molecules are located on Chr. 17 and thus are not included in the *Empsc1* locus determined in this study. In our experimental infections of mice, there was no statistically significant correlation between the number of mature protoscoleces and whole liver weight at 16 weeks p.i. measured as an index of metacestode growth in size (data not shown). These findings suggest that the metacestode growth of *E. multilocularis* is regulated, at least in part, by host factors different from those affecting protoscolex development. As generally recognized, mature protoscoleces are critical for the parasite to maintain its lifecycle, whereas the growth of the metacestode mass is intimately associated with the clinical outcome of AE. Hence, further investigations to identify QTLs responsible for metacestode growth in murine models may experimentally demonstrate the association between MHC genotypes and disease severity that was suggested in AE cases.

Table 3
List of candidate genes for *Emcys1* and *Empsc1*of *Mus musculus*.

Gene symbol	Gene function	cM
Emcys1 candidates on Chr. 6		
D6Mit188	Flanking marker	32.53
Cd207	CD 207 antigen	35.94
Trh	Thyrotropin releasing hormone	41.03
Il5ra	Interleukin 5 receptor, alpha	49.19
Vhlh	Von Hippel-Lindau syndrome homolog	52.81
Ghrl	Ghrelin	52.84
Hrh1	Histamine receptor H 1	53.05
D6Mit150		53.75
8-Mar	Membrane-associated ring finger (C3HC4) 8	53.78
Cxcl12	Chemokine (C-X-C motif) ligand 12	54.81
C3ar1	Complement component 3a receptor 1	57.93
Cd163	CD163 antigen	59.13
C1rl	Complement component 1, r subcomponent-like	59.16
C1r	Complement component 1, r subcomponent	59.16
C1s	Complement component 1, s subcomponent	59.17
Cd4	CD4 antigen	59.17
Lag3	Lymphocyte-activation gene 3	59.17
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	59.32
D6Mit254		59.32
Fgf23	Fibroblast growth factor 23	61.92
Klrb1c	Killer cell lectin-like receptor subfamily B member 1C	63.09
Cd69	CD69 antigen	63.21
Clec7a	C-type lectin domain family 7, member a	63.34
D6Mit15	Flanking marker	77.70
Empsc1 candidates on Chr. 1		
D1Mit445	Flanking marker	58.27
Ptprc	Protein tyrosine phosphatase, receptor type, C	60.73
Rgs1, 2, 13	Regulator of G-protein signaling 1, 2, 13	62.56
Rgs18	Regulator of G-protein signaling 18	62.99
Pla2g4a	Phospholipase A2, group IVA (cytosolic, calcium-dependent)	63.51
Ptgs2	Prostaglandin-endoperoxide synthase 2	63.84
Rgs8	Regulator of G-protein signaling 8	65.41
Rgsl1, 2	Regulator of G-protein signaling like 1, 2	65.43
Rgs16	Regulator of G-protein signaling 16	65.43
Abl2	V-abl Abelson murine leukemia viral oncogene 2 (arg, Abelson-related gene)	67.71
D1Mit14		67.71
Fasl	Fas ligand (TNF superfamily, member 6)	69.95
Sele	Selectin, endothelial cell	71.35
Sell	Selectin, lymphocyte	71.37
Selp	Selectin, platelet	71.42
Tiprl	TIP41, TOR signaling pathway regulator-like (*S. cerevisiae*)	72.55
Rgs4, 5	Regulator of G-protein signaling 4, 5	76.84
Atf6	Activating transcription factor 6	76.96
Fcgr2b	Fc receptor, IgG, low affinity IIb	78.02
Fcgr3	Fc receptor, IgG, low affinity III	78.80
Fcer1g	Fc receptor, IgE, high affinity I, gamma polypeptide	79.23
Slamf1	Signaling lymphocytic activation molecule family member 1	79.54
Cd48	CD48 antigen	79.54
Ncstn	Nicastrin	79.54
Crp	C-reactive protein, petaxin related	80.13
Fcer1a	Fc receptor, IgE, high affinity I, alpha polypeptide	80.33
D1Mit355	Flanking marker	80.36

cM, centiMorgan; Chr., chromosome.

The database available at the MGI website (http://www.informatics.jax.org/strains_SNPs.shtml) provides information on the strain-specific genomic features of mice. In the present investigation, however, only limited numbers of genomic differences were found between D2 and B6 strains on the identified QTLs (nine and 18 for *Emcys1* and *Empsc1*, respectively), all of which were located outside the coding region (data not shown). Since the database will be extended regularly, it might become useful to further narrow down the candidate genes in the future.

Using congenic techniques, we are currently attempting to generate mice strains that harbor QTLs from one selection line on the opposite line to investigate whether each allele has a different effect on the phenotype. At the same time, several approaches are currently being employed to identify candidate genes located on *Emcys1* and *Empsc1*. Some of these approaches include comparisons of gene expression levels of D2 and B6 mice in response to *E. multilocularis* infection using microarray and next-generation RNA sequencing technologies. This analytical combination that includes QTL mapping and gene expression profiles has proven useful in the selection of candidate genes (Wayne and McIntyre, 2002; Rennie et al., 2008; Ahn et al., 2010; Stark et al., 2010).

A lack of existing comprehensive information on the interplay between parasite and intermediate host makes it difficult to focus on certain genes responsible for resistance/susceptibility to *E. multilocularis* infection. However, our study provides several important conclusions. First, using QTL analysis, we were able to localize chromosomal sites where the allelic differences in genes present in D2 and B6 mice strongly affect mouse susceptibility to *E. multilocularis* infection. Second, multiple QTLs were detected

on different Chrs., indicating that susceptibility to infection is complex and is determined by multiple host genes. Additionally, larval establishment and development in mouse livers are controlled by distinct QTLs, indicating that there are different host factors interplaying with parasites at each developmental stage. Further identification of responsible genes located on the identified QTLs could lead to the development of effective disease prevention and control strategies, for example, an intensive screening and clinical follow-up of genetically high-risk groups for AE infection.

Acknowledgements

The first author was supported by a research grant fellowship from the Japanese Society for the Promotion of Science (JSPS) for young scientists. This work was financially supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) Grant-in-Aid for Scientific research (B) #20380164, the Global Center of Excellence (COE) Program, "Establishment of International Collaboration Centers for Zoonosis Control" from MEXT, and the Academic Frontier Project for Private Universities from MEXT.

References

Ahn, S.H., Deshmukh, H., Johnson, N., Cowell, L.G., Rude, T.H., Scott, W.K., Nelson, C.L., Zaas, A.K., Marchuk, D.A., Keum, S., Lamlertthon, S., Sharma-Kuinkel, B.K., Sempowski, G.D., Fowler, V.G., 2010. Two genes on A/J chromosome 18 are associated with susceptibility to *Staphylococcus aureus* infection by combined microarray and QTL analyses. PLoS Pathog. 6, e1001088.

Ali-Khan, Z., Siboo, R., 1981. *Echinococcus multilocularis*: distribution and persistence of specific host immunoglobulins on cysts membranes. Exp. Parasitol. 51, 159–168.

Bauder, B., Auer, H., Schilcher, F., Gabler, C., Romig, T., Bilger, B., Aspöck, H., 1999. Experimental investigations on the B and T cell immune response in primary alveolar echinococcosis. Parasite Immunol. 21, 409–421.

Beraldi, D., McRae, A.F., Gratten, J., Pilkington, J.G., Slate, J., Visscher, P.M., Pemberton, J.M., 2007. Quantitative trait loci (QTL) mapping of resistance to strongylus and coccidia in the free-living Soay sheep (*Ovis aries*). Int. J. Parasitol. 37, 121–129.

Brehm, K., 2010. The role of evolutionarily conserved signalling systems in *Echinococcus multilocularis* development and host–parasite interaction. Med. Microbiol. Immunol. 199, 247–259.

Breijo, M., Anesetti, G., Martínez, L., Sim, R.B., Ferreira, A.M., 2008. *Echinococcus granulosus*: the establishment of the metacestode is associated with control of complement-mediated early inflammation. Exp. Parasitol. 118, 188–196.

Craig, P.S., Rogan, M.T., Allan, J.C., 1996. Detection, screening and community epidemiology of taeniid cestode zoonoses: cystic echinococcosis, alveolar echinococcosis and neurocysticercosis. Adv. Parasitol. 38, 169–250.

Eiermann, T.H., Bettens, F., Tiberghien, P., Schmitz, K., Beurton, I., Bresson-Hadni, S., Ammann, R.W., Goldmann, S.F., Vuitton, D.A., Gottstein, B., Kern, P., 1998. HLA and alveolar echinococcosis. Tissue Antigens 52, 124–129.

Förster, S., Günthel, D., Kiss, F., Brehm, K., 2011. Molecular characterization of a serum-responsive, DAF-12-like nuclear hormone receptor of the fox-tapeworm *Echinococcus multilocularis*. J. Cell Biochem. 112, 1630–1642.

Gelmedin, V., Caballero-Gamiz, R., Brehm, K., 2008. Characterization and inhibition of a p38-like mitogen-activated protein kinase (MAPK) from *Echinococcus multilocularis*: antiparasitic activities of p38 MAPK inhibitors. Biochem. Pharmacol. 76, 1068–1081.

Gelmedin, V., Spiliotis, M., Brehm, K., 2010. Molecular characterisation of MEK1/2- and MKK3/6-like mitogen-activated protein kinase kinases (MAPKK) from the fox tapeworm *Echinococcus multilocularis*. Int. J. Parasitol. 40, 555–567.

Godot, V., Harraga, S., Beurton, I., Tiberghien, P., Sarciron, E., Gottstein, B., Vuitton, D.A., 2000. Resistance/susceptibility to *Echinococcus multilocularis* infection and cytokine profile in humans. II. Influence of the HLA B8, DR3, DQ2 haplotype. Clin. Exp. Immunol. 121, 491–498.

Gottstein, B., Felleisen, R., 1995. Protective immune mechanisms against the metacestode of *Echinococcus multilocularis*. Parasitol. Today 11, 320–326.

Gottstein, B., Wittwer, M., Schild, M., Merli, M., Leib, S.L., Müller, N., Müller, J., Jaggi, R., 2010. Hepatic gene expression profile in mice perorally infected with *Echinococcus multilocularis* eggs. PLoS One 5, e9779.

Hildreth, M.B., Granholm, N.H., 2003. Effect of mouse strain variations and cortisone treatment on the establishment and growth of primary *Echinococcus multilocularis* hydatid cysts. J. Parasitol. 89, 493–495.

Hillebrandt, S., Wasmuth, H.E., Weiskirchen, R., Hellerbrand, C., Keppeler, H., Werth, A., Schirin-Sokhan, R., Wilkens, G., Geier, A., Lorenzen, J., Köhl, J., Gressner, A.M., Matern, S., Lammert, F., 2005. Complement factor 5 is a quantitative trait gene that modifies liver fibrogenesis in mice and humans. Nat. Genet. 37, 835–843.

Iraqi, F.A., Behnke, J.M., Menge, D.M., Lowe, A.M., Teale, A.J., Gibson, J.P., Baker, L.R., Wakelin, D.R., 2003. Chromosomal regions controlling resistance to gastrointestinal nematode infections in mice. Mamm. Genome 14, 184–191.

Kassis, A.I., Tanner, C.E., 1977. Host serum proteins in *Echinococcus multilocularis*: complement activation via the classical pathway. Immunology 33, 1–9.

Konrad, C., Kroner, A., Spiliotis, M., Zavala-Góngora, R., Brehm, K., 2003. Identification and molecular characterisation of a gene encoding a member of the insulin receptor family in *Echinococcus multilocularis*. Int. J. Parasitol. 33, 301–312.

Korstanje, R., Eriksson, P., Samnegård, A., Olsson, P.G., Forsman-Semb, K., Sen, S., Churchill, G.A., Rollins, J., Harris, S., Hamsten, A., Paigen, B., 2004. Locating *Ath8*, a locus for murine atherosclerosis susceptibility and testing several of its candidate genes in mice and humans. Atherosclerosis 177, 443–450.

Lander, E., Kruglyak, L., 1995. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat. Genet. 11, 241–247.

Manly, K., Cudmore, R.J., Meer, J., 2001. Map Manager QTX, cross-platform software for genetic mapping. Mamm. Genome 12, 930–932.

Matsumoto, J., Kouguchi, H., Oku, Y., Yagi, K., 2010. Primary alveolar echinococcosis: course of larval development and antibody responses in intermediate host rodents with different genetic backgrounds after oral infection with eggs of *Echinococcus multilocularis*. Parasitol. Int. 59, 435–444.

Nakaya, K., Nakao, M., Ito, A., 1997. *Echinococcus multilocularis*: mouse strain difference in hydatid development. J. Helminthol. 71, 53–56.

Peters, L.L., Robledo, R.F., Bult, C.J., Churchill, G.A., Paigen, B.J., Svenson, K.L., 2007. The mouse as a model for human biology: a resource guide for complex trait analysis. Nat. Rev. Genet. 8, 58–69.

Rennie, C., Hulme, H., Fisher, P., Halp, L., Agaba, M., Noyes, H.A., Kemp, S.J., Brass, A., 2008. A systematic, data-driven approach to the combined analysis of microarray and QTL data. Dev. Biol. (Basel) 132, 293–299.

Spiliotis, M., Kroner, A., Brehm, K., 2003. Identification, molecular characterization and expression of the gene encoding the epidermal growth factor receptor orthologue from the fox-tapeworm *Echinococcus multilocularis*. Gene 323, 57–65.

Spiliotis, M., Tappe, D., Brückner, S., Mösch, H.U., Brehm, K., 2005. Molecular cloning and characterization of Ras- and Raf-homologues from the fox-tapeworm *Echinococcus multilocularis*. Mol. Biochem. Parasitol. 139, 225–237.

Spiliotis, M., Konrad, C., Gelmedin, V., Tappe, D., Brückner, S., Mösch, H.U., Brehm, K., 2006. Characterisation of EmMPK1, an ERK-like MAP kinase from *Echinococcus multilocularis* which is activated in response to human epidermal growth factor. Int. J. Parasitol. 36, 1097–1112.

Stark, J.M., Barmada, M.M., Winterberg, A.V., Majumber, N., Gibbons, W.J., Stark, M.A., Sartor, M.A., Medvedovic, M., Kolls, J., Bein, K., Mailaparambil, B., Krueger, M., Heinzmann, A., Leikauf, G.D., Prows, D.R., 2010. Genomewide association analysis of respiratory syncytial virus infection in mice. J. Virol. 84, 2257–2269.

Suzuki, T., Ishih, A., Kino, H., Muregi, F.W., Takabayashi, S., Nishikawa, T., Takagi, H., Terada, M., 2006. Chromosomal mapping of host resistance loci to *Trichinella spiralis* nematode infection in rats. Immunogenetics 58, 26–30.

Vuitton, D.A., 2003. The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? Acta Trop. 85, 119–132.

Vuitton, D.A., Gottstein, B., 2010. *Echinococcus multilocularis* and its intermediate host: a model of parasite–host interplay. J. Biomed. Biotechnol. 2010, 923193.

Wayne, M.L., McIntyre, L.M., 2002. Combining mapping and arraying: An approach to candidate gene identification. Proc. Natl. Acad. Sci. USA 99, 14903–14906.

Webster, G.A., Cameron, T.W.M., 1961. Observations on experimental infections with *Echinococcus* in rodents. Can. J. Zool. 39, 877–891.

Williams-Blangero, S., Vandeberg, J.L., Subedi, J., Jha, B., Dyer, T.D., Blangero, J., 2008. Two quantitative trait loci influence whipworm (*Trichuris trichiura*) infection in a Nepalese population. J. Infect. Dis. 197, 1198–1203.

Yamashita, J., Ohbayashi, M., Kitamura, Y., Suzuki, K., Okugi, M., 1958. Studies on *echinococcosis* VIII. Experimental *Echinococcosis multilocularis* in various rodents; especially on the difference of susceptibility among uniform strains of the mouse. Jpn. J. Vet. Res. 6, 135–159.

Yang, Y.R., Ellis, M., Sun, T., Li, Z., Liu, X., Vuitton, D.A., Bartholomot, B., Giraudoux, P., Craig, P.S., Boufana, B., Wang, Y., Feng, X., Wen, H., Ito, A., McManus, D.P., 2006. Unique family clustering of human echinococcosis cases in a Chinese community. Am. J. Trop. Med. Hyg. 74, 487–494.

Yoshida, Y., Makita, Y., Heida, N., Asano, S., Matsushima, A., Ishii, M., Mochizuki, Y., Masuya, H., Wakana, S., Kobayashi, N., Toyoda, T., 2009. PosMed (Positional Medline): prioritizing genes with an artificial neural network comprising medical documents to accelerate positional cloning. Nucleic Acids Res. 37, W147–152.

Zavala-Góngora, R., Kroner, A., Wittek, B., Knaus, P., Brehm, K., 2003. Identification and characterisation of two distinct Smad proteins from the fox-tapeworm *Echinococcus multilocularis*. Int. J. Parasitol. 33, 1665–1677.

Zavala-Góngora, R., Kroner, A., Bernthaler, P., Knaus, P., Brehm, K., 2006. A member of the transforming growth factor-beta receptor family from *Echinococcus multilocularis* is activated by human bone morphogenetic protein 2. Mol. Biochem. Parasitol. 146, 265–271.

Zavala-Góngora, R., Derrer, B., Gelmedin, V., Knaus, P., Brehm, K., 2008. Molecular characterisation of a second structurally unusual AR-Smad without an MH1 domain and a Smad4 orthologue from *Echinococcus multilocularis*. Int. J. Parasitol. 38, 161–176.

Zhang, S., Penfornis, A., Harraga, S., Chabod, J., Beurton, I., Bresson-Hadni, S., Tiberghien, P., Kern, P., Vuitton, D.A., 2003. Polymorphisms of the TAP1 and TAP2 genes in human alveolar *echinococcosis*. Eur. J. Immunogenet. 30, 133–139.

Experimental Parasitology 128 (2011) 50–56

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Echinococcus multilocularis: Purification and characterization of glycoprotein antigens with serodiagnostic potential for canine infection

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ARTICLE INFO

Article history:
Received 17 September 2010
Received in revised form 31 January 2011
Accepted 1 February 2011
Available online 12 February 2011

Keywords:
Echinococcus multilocularis
Alveolar echinococcosis
Serodiagnosis
Immunogenic protein
Emgp-89
Canine echinococcosis

ABSTRACT

We show that a conventionally purified glycoprotein component of *Echinococcus multilocularis* protoscolex, designated as Emgp-89, may be useful as a serodiagnostic antigen for detecting *E. multilocularis* infection in dogs domesticated in endemic areas. Emgp-89 was obtained from the parasite material by a simple procedure using Con A-agarose and subsequent gel filtration chromatography. The purified fraction showed a molecular weight of >4000 kDa upon gel filtration and reacted with a series of lectins that specifically bind to mannose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine. Subsequently, serodiagnostic performance of Emgp-89 was evaluated through enzyme-linked immunosorbent assays (ELISAs) by using sera from normal, domestic dogs and dogs infected with other helminths. Emgp-89 positively reacted with all 16 serum samples from *E. multilocularis*-infected dogs, thus showing that this antigen is highly sensitive. On the other hand, the specificity of Emgp-89-based ELISA, determined using 41 serum samples from dogs infected with other helminths, was relatively low (83%). As an attempt to improve the specificity of Emgp-89-based ELISA, we pretreated Emgp-89 with proteinase K or sodium periodate, expecting that these treatments would enable discrimination of true positives from false positives. The ELISA value increased after treatment with sodium periodate in most false-positive samples, whereas significant decreases were observed in sera from all dogs infected with *E. multilocularis*. Further evaluation of this antigen should be performed using sera from dogs infected with closely-related parasites, including taeniid cestodes, which are expected to prove that this serodiagnostic system is sufficiently specific for clinical and field applications.

1. Introduction

Echinococcus multilocularis, which causes the parasitic disease alveolar echinococcosis (AE), is widely distributed throughout the Northern hemisphere, especially central Europe, North America, China, and the north island of Japan (Rausch, 1995; Torgerson and Budke, 2003). In human AE, infection is caused by accidental ingestion of infective eggs. Each egg releases an oncosphere upon stimulation by bile in the intestinal lumen. The oncosphere migrates to the liver via the circulatory system and forms mass of small cysts. Ten or more years after the initial parasitic infection, the metacestodes proliferate unrestrictedly in the liver and other organs and form the tumor-like mass, which might cause organ dysfunction. The prevalence of *E. multilocularis* infection among humans is generally low; however, AE can be highly lethal because of the unlimited proliferation and metastasis of the parasitic lesions, unless appropriate treatment is administered.

With the exception of highly AE endemic areas such as western China (Budke et al., 2004, 2005), the major definitive hosts of *E. multilocularis* are wild foxes; dogs are not considered to play an important role in the natural transmission of the parasite. However, dogs are highly susceptible to experimental infection with the adult parasite, suggesting that accidentally infected dogs could be an important source of AE infection in humans because of their close contact with their owners. Thus, we sought to establish measures to reduce the risk of AE transmission from dogs to humans.

The development of novel diagnostic methods for the detection of adult *E. multilocularis* infection in dogs is an important strategy for the evaluation of AE prevalence and for the development of surveillance and control programs for canine echinococcosis (Eckert et al., 2001). The identification of the *Echinococcus* spp. causing infection in the definitive hosts is difficult because the eggs of all *Echinococcus* and *Taenia* species are morphologically indistinguishable, and characteristic small segments of *Echinococcus* spp. may be

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doi:10.1016/j.exppara.2011.02.002

absent from the feces or may be overlooked. Several diagnostic methods have been developed and used in combination with the above mentioned parasitologic examinations for the detection of the *Echinococcus* infection in living dogs, including immunologic detection of the excretory/secretory component released from the parasite into host feces (Deplazes and Eckert, 1996; Nonaka et al., 1996; Sakashita et al., 1995). However, the sensitivity of this technique is highly variable owing to inconsistent antigen excretion (Wachira et al., 1990). Furthermore, the handling of fecal samples is accompanied by the risk of accidental infection. Serological diagnosis would overcome these problems and would provide important data to trace the history of the parasitic infection in definitive hosts. However, little information is available about immune responses in dogs during the course of *E. multilocularis* infection because the handling of definitive hosts infected with the parasite is associated with the risk of AE infection.

Recently, we found several immunogenic antigens that specifically reacted with sera from dogs infected with *E. multilocularis* (Katoh et al., 2008; Kouguchi et al., 2007). An unknown component containing glycoprotein was found by two-dimensional western blot (2D-WB) analysis of a cell extract of *E. multilocularis* protoscoleces (Kouguchi et al., 2010). The unknown component significantly reacted with sera from dogs experimentally infected with *E. multilocularis* but did not react with sera from healthy dogs or from domesticated dogs. These results encouraged us to explore the use of this sensitive and specific glycoprotein antigen in the serodiagnosis of definitive hosts infected with *E. multilocularis*. In the present study, experimental infections were induced in a safe facility designed for the experimental infection of dogs with *E. multilocularis*, yielding materials for subsequent purification and characterization of the immunologically dominant glycoprotein recognized by sera from the infected dogs.

2. Materials and methods

2.1. Parasite materials

Echinococcus multilocularis (Nemuro strain) was obtained from a dog-cotton rat life cycle maintained at the Hokkaido Institute of Public Health. Approximately 50 eggs were administered to a cotton rat. Protoscoleces were collected from developed cysts of cotton rats 10–14 months after infection and washed 7 times with 10 mM phosphate buffer (pH 7.4) containing 137 mM NaCl and 2.7 mM KCl (PBS) with penicillin G (500 IU/ml) and streptomycin (1 mg/ml).

2.2. Serum samples

Fifteen sera from dogs experimentally infected with *E. multilocularis* and one serum from dog naturally infected with *E. multilocularis* were used in this study. The cyst materials, which included approximately 5×10^5 *E. multilocularis* protoscoleces, were used for the experimental infection of each dog. Serum samples were collected from infected dogs (3–16 months old males) each week following infection, and sera were stored individually at −30 °C until use. Unless otherwise specified, the sera collected 42 days after infection were used in serological experiments. All animal experiments were done in line with our institutional guide lines after permission of the Animal Research Committee. All experiments were performed in a specially designed safety facility (biosafety level 3) in the Hokkaido Institute of Public Health, Sapporo, Japan.

Three types of negative control sera were used in this study. Twenty sera from healthy dogs (4 months–6 years old) were purchased from Sankyo Lab Service Co., Ltd. (Sapporo, Japan). Twenty sera from domestic dogs (1–13 years old) living in an unendemic area were kindly provided by Dr. K. Sawashima (Sawashima Veterinary Clinic, Kobe, Japan). *Dirofilaria immitis* infection and parasite eggs were not detected in any of these dogs. Forty-one sera from dogs naturally infected with *Dipylidium caninum*, *Ancylostoma caninum*, *D. immitis*, *Toxocara canis*, *Trichuris vulpis* or *Taenia hydatigena* were kindly provided by Professor S. Nogami (Nihon University, Fujisawa, Japan).

2.3. Purification of E. multilocularis glycoprotein component (Emgp-89)

Approximately 1.4 ml of the washed protoscoleces was transferred to a polystyrene test tube (17 × 100 mm) and 5 ml PBS supplemented with 2.4% Triton X-100. Following gentle shaking for 3 min at room temperature, the suspension was sonicated 6 times for 10 s with a probe-type ultrasonic generator (UCD-130, Tosho electronic co.) at 4 °C to obtain a crude extract of protoscoleces. The crude extract was centrifuged for 15 min at 3500 rpm and the clarified supernatant was dialyzed against distilled water overnight. The dialyzed solution was separated into aliquots of approximately 5 ml in a small vial and lyophilized.

The lyophilized crude extract was dissolved in 8 ml of 20 mM Tris–HCl buffer (pH 7.8) containing 0.15 M NaCl and an appropriate amount of protease inhibitor cocktail (complete EDTA free, Roche). The dissolved crude extract was mixed with 2 ml of Con A-agarose (J-Oil mills, Inc. Tokyo, Japan), incubated at 4 °C overnight on a rotator, and poured into a column. The resin was washed with 10 volumes of 20 mM Tris–HCl buffer (pH 7.8) containing 0.15 M NaCl. Absorbed proteins were eluted with 5 volumes of 20 mM Tris–HCl buffer (pH 7.8) containing 250 mM D-mannose. The affinity fractions were concentrated to 400 µl with Centricon YM 10 ultrafiltration units. Two hundred microliters of sample was applied to a Hiload Superdex 200HR column fitted on AKTA explorer (GE healthcare) equilibrated with PBS containing 0.1% octyl glucoside or 0.05 M carbonate buffer (pH 9.6) at a flow rate of 0.3 ml/min. Each fraction was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and western blot using a serum pool from dogs infected with *E. multilocularis*, as described below. Elution positions of thyroglobulin (669 kDa), aldolase (158 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa) were determined in a separate run under identical conditions. Superose 6 10/300GL (GE healthcare) column was used to determine purity of the samples under identical condition described above.

2.4. Western blotting

SDS–PAGE-separated proteins were electroblotted onto a polyvinylidene fluoride (PVDF) membrane and incubated in blocking buffer (PBS containing 10% skim milk and 0.1% Tween 20) for 1 h. The membrane was incubated with a serum pool from dogs infected with *E. multilocularis* (1:400 dilution in blocking buffer) for 1 h. After the membrane was washed, it was incubated with anti-dog IgG-alkaline phosphatase (AP) conjugate (1:3000 dilution in blocking buffer). Bound antibodies were detected with a BCIP/NBT immuno-detection kit (NEN Life Science).

2.5. ELISA

Specific serum antibodies to Emgp-89 or fractions of gel filtration chromatography were measured by an indirect ELISA as described previously (Matsumoto et al., 1998). A 96-well ELISA plate (MICROLON600; Greiner Bio-one, Frickenhausen, Germany) was coated with the antigen preparation overnight at 4 °C. The antigens were diluted in 0.05 M carbonate buffer (pH9.6) to a concentration of 25 µg/ml. Protein concentration was calculated by optical density at 280 nm with BSA as a standard (0.7 OD at 280 nm = 1 mg/ml). Each well was washed with PBS containing

0.1% Tween 20 (PBS/Tween), blocked with blocking buffer (1% (w/v) BSA in PBS/Tween) for 1 h at 37 °C, and washed with PBS/Tween. In some experiments, antigen-coated wells were treated with either 100 mM $NaIO_4$ in 40 mM sodium acetate buffer (pH 5.5) or 50 μg of proteinase K/ml for 1 h at 37 °C to disrupt the carbohydrate or protein epitope, respectively, as previously described (Walker et al., 2004). Each well was washed with PBS/Tween, blocked with dilution buffer (0.5% (w/v) BSA, 0.5% (w/v) casein in PBS/Tween) for 1 h at 37 °C, and washed with PBS/Tween.

The wells were reacted with sample sera diluted 1:100 with dilution buffer for 1 h at 37 °C. The wells were washed with PBS/Tween, and incubated with horseradish peroxidase-conjugated rabbit anti-dog IgG (whole molecule; Sigma–Aldrich Co., St. Louis, MO) at a dilution of 1:2500 in dilution buffer for 1 h at 37 °C. Following the final wash with PBS/Tween, substrate solution containing 0.04% o-phenylenediamine and 0.006% H_2O_2 in 100 mM citrate phosphate buffer (pH 5.0) was applied to each well. The plate was incubated for 6 min at room temperature and the optical density was read at 492 nm.

2.6. Lectin ELISA

For the detection of Emgp-89 lectin-binding sites, the following biotinylated lectins were used: *Maackia amurensis* agglutinin (MAM) affinity for *N*-acetyl neuramic acid, *Ulex europaeus* agglutinin I (UEA-I), *Canavalia ensiformis* agglutinin (Con A), *Arachis hypogaea* agglutinin (PNA), *Erythrina cristagalli* agglutinin (ECA), *Triticum vulgaris* agglutinin (WGA), and Glycine max agglutinin (SBA). ELISA plates were coated with Emgp-89 as described above. Nonspecific sites were blocked with 25 mM Tris–HCl buffer containing 137 mM NaCl, 2.7 mM KCl, and 0.3% Tween 20 (pH 7.4, TBST) for 2 h at room temperature. Plates were incubated for 1 h with lectins diluted in 25 mM Tris HCl buffer (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl and 1 mM $CaCl_2$ at a final concentration of 5 μg/ml. Parallel to all lectin ELISAs, control incubations in the presence of 500 mM of the corresponding inhibitory sugar were performed. These included *N*-acetyl neuramic acid (NANA) for MAM, D-fucose (Fuc) for UEA-I, D-mannose (Man) for Con A, D-galactose (Gal) for PNA and ECA, *N*-acetyl glucosamine (GlcNAc) for WGA, and *N*-acetyl galactosamine (GalNAc) for SBA. Plates were washed 3 times for 10 min with TBST, and were incubated with a streptavidin-peroxidase conjugate at room temperature for 1 h at a dilution of 1:2500 in TBST containing 0.05% BSA. Plates were washed 3 times for 5 min with TBST and incubated with substrate solution for 6 min at room temperature. Their optical density was read at 492 nm.

2.7. Data analysis

The cutoff value was calculated for each test from the mean absorbance value obtained for the 20 healthy dogs plus 3 standard deviations. The following definitions were used to calculate the corresponding diagnostic parameters: true-positive values (tp), sera from dogs infected with *E. multilocularis* showing positive readings; false-negative values (fn), sera from dogs infected with *E. multilocularis* showing negative readings; false-positive values (fp), sera from healthy dogs or dogs without *E. multilocularis* infection showing positive readings; true-negative values (tn), sera from healthy dogs or dogs without *E. multilocularis* infection showing negative readings; sensitivity, $tp \times 100/(tp + fn)$; specificity, $tn \times 100/(tn + fp)$.

3. Results

3.1. Purification of Emgp-89

Crude extract of *E. multilocularis* protoscoleces was purified by Con A-agarose column affinity chromatography. The fractions including crude glycoproteins were concentrated and further purified by Superdex 200HR column gel filtration as 1-ml individual fractions. As illustrated in Fig. 1A, a major peak was eluted at an

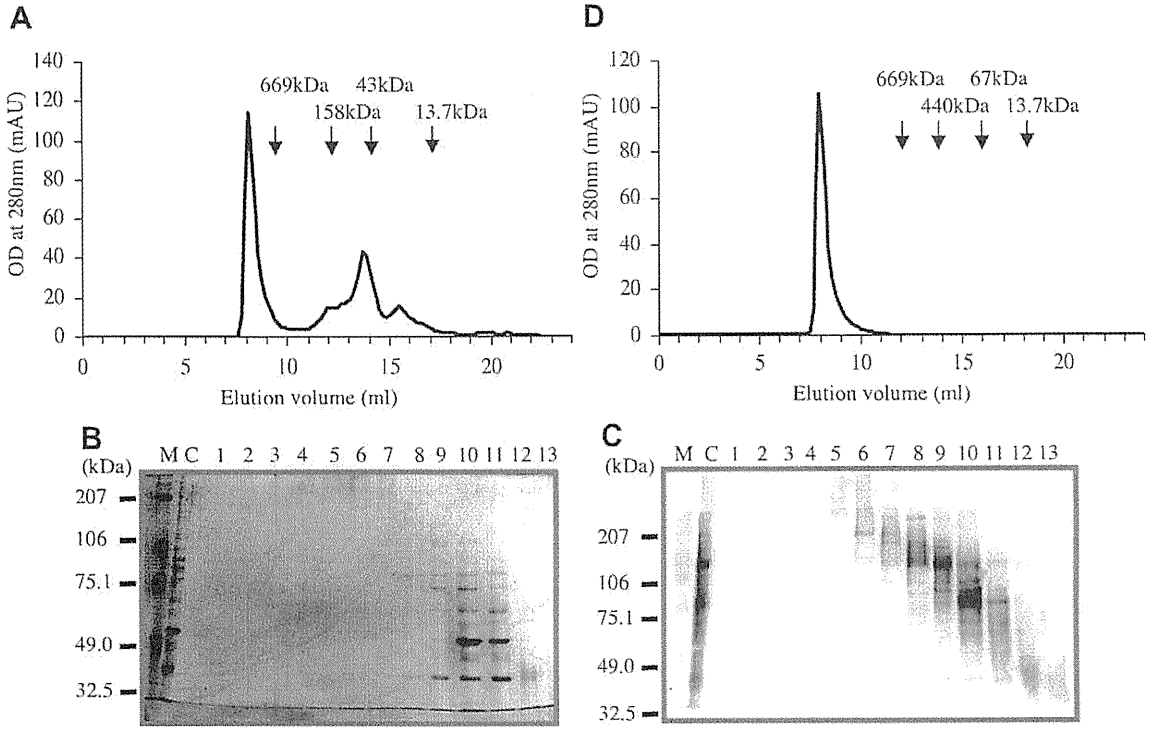

Fig. 1. Gel filtration chromatography of the ConA-agarose fraction using Superdex 200HR 10/20 column (A). One millilitre fractions were collected in the range of 4–17 ml. Each fraction was applied to SDS–PAGE with 7.5% gel (B) and western blot analysis using the serum pool from dogs infected with *E. multilocularis* (C). Lane M, molecular size marker; lane C, Con A-agarose fraction; 1–13, fractions in panel A. Fraction 5 in panel A was concentrated and applied to Superose 6 10/300GL column gel filtration chromatography (D). The elution positions of molecular markers are indicated by arrows.

elution volume of 7.5–9.5 ml, which corresponded to the void volume on the Superdex 200HR column. Fractions eluted from 4 to 7 ml range (corresponding to fractions 1–4) showed no visible signals on SDS–PAGE gel or by western blot analysis using pooled sera from dogs infected with *E. multilocularis* (Fig. 1B and C). Fraction 5 corresponded to a major peak and showed a weak and smeared band at approximately 207 kDa near the top of the SDS–PAGE running gel. Fraction 5 was concentrated and applied to Superose 6 10/300 column with exclusion limit of 4000 kDa. The fraction 5 was eluted as a single sharp peak at the position of void volume. This indicated that the fraction 5 contained only very large molecule (s) (Fig. 1D).

In addition, fraction 5 showed a clear band on the western blot, extending from a position corresponding to the top of the running gel to the top of the stacking gel. Fraction 6 showed a similar banding profile to fraction 5 on SDS–PAGE but fraction 6 had a relatively strong band at 207 kDa as detected by western blot analysis (Fig. 1C, lane 6). Fractions 7 and 8 showed similar banding profiles as fractions 5 and 6 but strong band densities were detected from 106 kDa to the top of the membrane. Fractions 9–11 showed several bands with molecular masses of 106, 90, 80, 75, 68, 52, and 42 kDa on SDS–PAGE, and ladder bands were observed at 35–207 kDa on western blot. Fractions 12 and 13 showed a weak band at approximately 42 kDa on SDS–PAGE and showed weak and smeared bands at 32–80 kDa on western blot.

3.2. Screening of gel filtration fractions by ELISA

In order to determine which fraction contained the promising antigen for use in the serodiagnosis of infected dogs, ELISA was performed using each fraction with 11 sera from dogs infected with *E. multilocularis* and with 9 control sera (2 sera from domestic dogs and 7 sera from other helminths-infected dogs) that showed a relatively high nonspecific reaction with the Con A-agarose fraction in the preliminary ELISA experiment. Of the 8 fractions assayed, fraction 5 showed the greatest specificity. Thus, we decided to use fraction 5 to develop an antigen for use in the serodiagnosis, and this was designated Emgp-89.

3.3. Evaluation of serodiagnositic value of Emgp-89 based-ELISA

As shown in Fig. 2, the serodiagnostic value of Emgp-89 was tested using sera from dogs experimentally infected with *E. multilocularis* (15 samples), dog naturally infected with *E. multilocularis* (1 sample), healthy dogs (20 samples), domestic dogs living in an unendemic area (20 samples), and dogs naturally infected with other helminths (41 samples). The Emgp-89-based-ELISA showed a positive reaction in all 16 of the sera collected from dogs infected with *E. multilocularis* based on a cutoff value of 0.25, the mean of healthy dog sera plus 3 standard deviations (SDs). Seven sera from dogs infected with other helminths and one serum from domestic dog showed ELISA values greater than 0.25 and were judged to be false-positives. From these result, the specificity was calculated as 90%. Sera from dogs infected with other helminths showed a relatively higher average value (0.20, average of 41 samples) than sera from healthy dogs (0.16, average of 20 samples) and domestic dogs (0.18, average of 20 samples). The average value of the sera from *E. multilocularis* infected-dogs was 0.47 (average of 16 samples).

3.4. Emgp-89 based-ELISA pretreated with proteinase K and $NaIO_4$

To determine whether the sera that reacted with Emgp-89 recognized the protein(s) or carbohydrate moieties, the ELISA antigen was pretreated with proteinase K or $NaIO_4$ before incubation with sera judged to be false positives, sera with absorbance values >0.23 (Fig. 2), or sera of dogs infected with *E. multilocularis* (Fig. 3). Treatment of Emgp-89 with either proteinase K or $NaIO_4$ resulted in a substantial reduction of reactivity with sera from dogs infected with *E. multilocularis*. In sera from dogs infected with *E. multilocularis*, the reduction reached a maximum of 43.7% and a minimum of 65.1% of control values (same samples in the absence of proteinase K) for proteinase K treatment. Similarly, the reduction reached a maximum of 26.9% and a minimum of 71.9% of control values (same samples in the absence of $NaIO_4$) for $NaIO_4$ treatment. In contrast, treatment with $NaIO_4$ resulted in a significant increase and/or had almost no effect on reactivity in the false-positive samples and serum samples sera with absorbance values >0.23 in Fig. 2 (ranging from 92.9% to 177.0% of control values), although a clear reduction was observed following proteinase K treatment (ranging from 66.3% to 78.5% of control values). An exception was a single cross-reactive sample from a domestic dog (No. 24 in Fig. 3) that showed significant reduction in reactivity following $NaIO_4$ treatment (44.0% of control value) but almost no effect was observed following proteinase K treatment (97.5% of control value).

3.5. Lectin ELISA

To obtain information on the type of carbohydrate residues present in the purified Emgp-89, lectin ELISAs were performed using a series of biotinylated lectins with affinities for selected carbohydrates (Fig. 4). Emgp-89 reacted with Con A, PNA, ECA, WGA and SBA. No reactivity was observed with MAM and UEA-I. Control incubations in the presence of 500 mM of inhibitory sugar exhibited an almost complete inhibition of the reaction between each

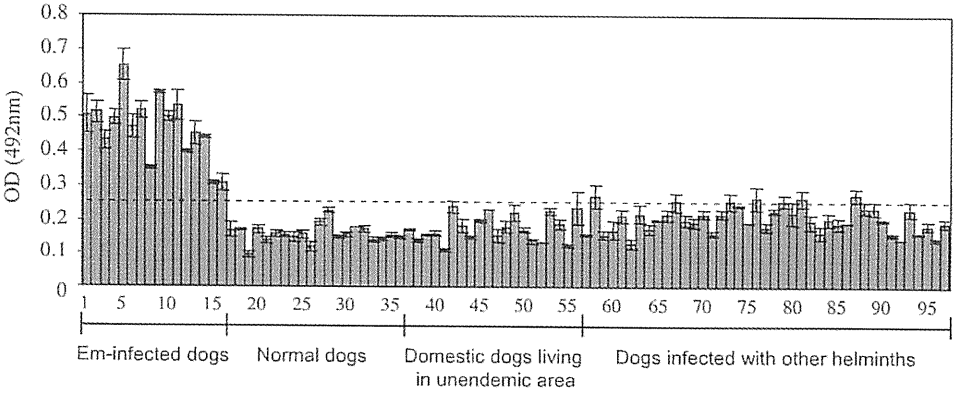

Fig. 2. Serological evaluation of Emgp-89 in ELISA using the sera from dogs infected with *E. multiloculars* (serum Nos. 1–16), healthy dogs (serum Nos. 17–36), domesticated dogs (serum Nos. 37–56), and dogs infected with the other helminths (serum Nos. 57–60 for *D. immitis*, 61–64 for *A. caninum*, 65–69 for *T. vulpis*, 70–73 for *T. canis*, 74–96 for *D. caninum* and 97 for *T. hydatigena*). Results represent the means of 2 independent experiments with different antigen lots, and error bars represent the standard deviations.

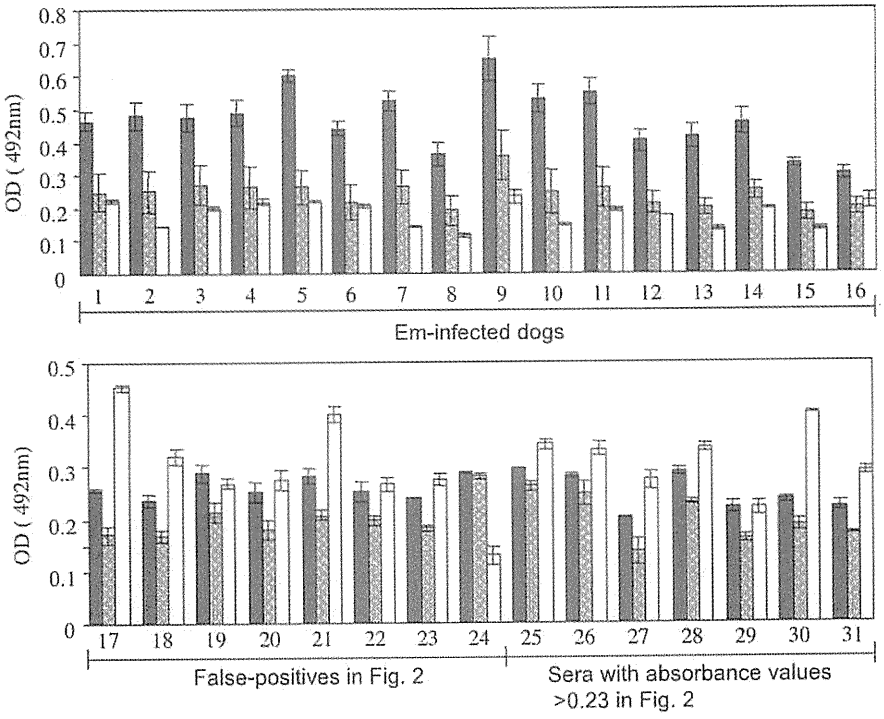

Fig. 3. Effect of pretreatment with proteinase K or $NaIO_4$ on Emgp-89 immunoreactivity. ELISA was performed with sera from dogs infected with *E. multilocularis* (Nos. 1–16), the sera judged as false-positives in Fig. 2 (Nos. 17–24) and the sera with absorbance value >0.23 in Fig. 2 (Nos. 25–31). The Emgp-89 was reacted with sera before (closed bars) or after treatment with proteinase K (gray bars) or $NaIO_4$ (open bars). Results represent the means of 2 independent experiments with different antigen lots, and error bars represent the standard deviations.

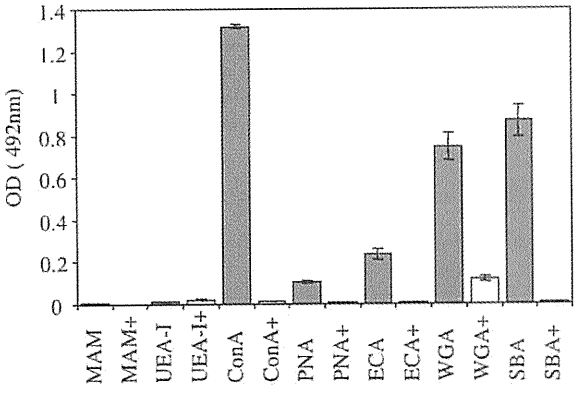

Fig. 4. Lectin reactivity of the Emgp-89. Binding of lectin was analyzed by ELISA with biotinylated lectins as illustrated in the panel. Closed columns indicate reactivity of lectins, and white columns indicate reactivity from identical experiments in the presence of 500 mM of the corresponding inhibitory sugar. Results represent the means of 2 independent experiments with different antigen lots, and error bars represent the standard deviations.

lectin and Emgp-89, which verified that the reactivity of these lectins were based on lectin-carbohydrate interactions.

3.6. Time course of specific antibody response against Emgp-89

We examined the time course of a specific antibody response against the Emgp-89 by using sera collected weekly from 5 infected dogs. In this experiment, 2 infected dogs were dewormed by oral administration of praziquantel 42 days after infection. The specific antibody level increased approximately 1 week after infection, and the levels reached a maximum approximately 5–6 weeks after infection in all dogs tested (Fig. 5). Furthermore, the antibody level remained slightly elevated 4 months after chemical deworming. Sera from other dogs infected with *E. multilocularis* showed an almost identical curve (data not shown).

During the course of *E. multilocularis* infection in dogs, the worms were naturally expelled from the infected animals by unknown mechanisms, probably due to physiologic or immunologic exclusion (Kapel et al., 2006; Matsumoto and Yagi, 2008; Thompson et al., 2006). The above mentioned findings suggest that *E. multilocularis* infection in dogs can be detected by Emgp-89-based serodiagnosis for up to 4 months after the parasite expulsion.

4. Discussion

In the present study, we successfully established a simple method for the purification of novel glycoprotein fraction, designated as Emgp-89, and evaluated its serolodiagnostic performance using sera from dogs infected with *E. multilocularis*. During the course of Emgp-89 purification, we characterized the serological properties of Con A-agarose fractions in the preliminary experiment (data not shown), and the Con A fraction appeared to have high reactivity with sera from dogs infected with *E. multilocularis*. However, the Con A fraction showed nonspecific reactions to some sera from healthy and domestic dogs (unpublished data), so we further purified the Con A fraction by gel filtration. The Emgp-89 was eluted at void volume and was detected by western blot analysis using a serum pool from dogs infected with *E. multilocularis* as a smeared band from the position corresponding to the top of the running gel to the stacking gel (Fig. 1C). In addition, the component was not sensitive to Coomassie brilliant blue staining (Fig. 1B). Similar characteristics were reported for mucin glycoproteins of other organisms (Montagne et al., 2000; Tanabe et al., 2007).

A similar Con A purification method was used to attempt to develop a sensitive diagnosis and quantitation of *Echinococcus granulosus* infection in human and animal hosts (Kamel et al., 2006). The Con A-purified glycoprotein fraction showed significant

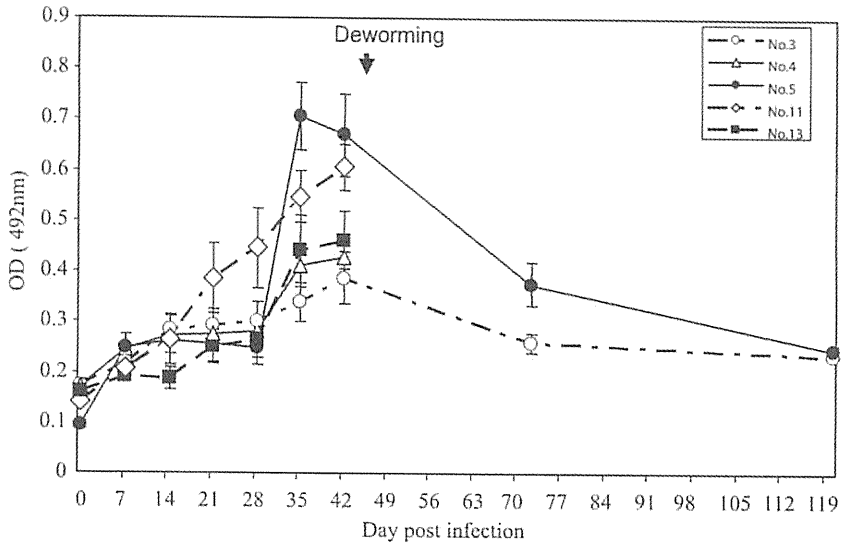

Fig. 5. Time course of antibody responses to Emgp-89 in the sera from dogs infected with *E. multilocularis*. Sera were collected weekly after oral administration of approximately 5×10^5 *E. multilocularis* protoscoleces. The values were determined by ELISA, as described in materials and methods. Serum numbers (3, 4, 5, 11, and 13) correspond to the sera in Fig. 2. Results represent the means of 2 independent experiments with different antigen lots, and error bars represent the standard deviations.

diagnostic efficacy, suggesting that the Con A purified fraction contained immunologically important components against a host infected with *Echinococcus* species. These data suggest that examination of components of the Con A fractions of *Echinococcus* metacestodes would facilitate the discovery of unique molecules that can be used for diagnosis or development of a practical vaccine.

In the evaluation of serodiagnostic performance of Emgp-89, this glycoprotein fraction showed 100% sensitivity in 16 sera from dogs infected with *E. multilocularis*. On the other hand, generally, the specificity of Emgp-89 was calculated as 90% when determined from the results of the sera from healthy, domestic dogs and dogs infected with other helminths. However, in the field, this test would be used for dogs infected by *E. multilocularis* versus those that have a high possibility of being naturally infected by helminths other than *E. multilocularis*. Therefore, we considered that the practical 'field specificity' of this test was relatively low, at 83%—34 out of 41 samples from dogs infected with helminths other than *E. multilocularis* showed negative values.

To improve the specificity of Emgp-89 based ELISA, we conducted a second discrimination test using $NaIO_4$ and proteinase K. Pretreatment with $NaIO_4$ and proteinase K showed an interesting difference between the ELISA profiles of sera from dogs infected with *E. multilocularis*, of serum samples with absorbance values >0.23 (Fig. 2), and of false-positive sera. ELISA values of sera from *E. multilocularis* infected-dogs were decreased by treatment with both $NaIO_4$ and proteinase K. However, ELISA values were significantly increased by treatment with $NaIO_4$ in most false-positive sera and sera with absorbance value >0.23 in Fig.2. This observation may indicate that disrupting the carbohydrate moiety of the Emgp-89 led to increased antibody accessibility to the Emgp-89 protein (s). This interpretation is consistent with our observation that proteinase K treatment decreased ELISA values of false-positive sera and sera with absorbance value >0.23 in Fig.2. As an exception, 1 sample showed a significantly reduced ELISA value after $NaIO_4$ treatment but almost no effect was observed after proteinase K treatment. These results suggest that sera showing cross-reactivity recognize either the carbohydrate moieties or Emgp-89 protein. Thus, ELISA performed after these pretreatments are an effective method to distinguish positives from false positives.

For evaluating the specificity of Emgp-89-based ELISA, we should have included sufficient number of serum samples obtained from dogs infected with closely-related species such as taeniid cestodes, which were not available in the present study. Several species of parasites belonging to the genus *Taenia* use dogs as suitable final hosts, and they are commonly found in dogs in some areas in the world. Therefore, the presence or absence of cross-reactivity of Emgp-89 against sera from dogs infected with taeniid species should essentially be tested before Emgp-89-based ELISA is used for clinical and field applications.

To our knowledge, studies concerning the development of serodiagnostic techniques for dogs with *E. multilocularis* infection are limited. Early attempts, including immunity in a dog by serodiagnosis with native and recombinant antigen from *Echinococcus* species, produced some significant results. Deplazes and Eckert (1996) used the Em2 antigen of *E. multilocularis* to diagnose parasitic infection in the final host. They showed that specific circulating antibodies against *E. multilocularis* Em2 could be detected by ELISA in 12–60% of approximately 400 foxes originating from populations infected with *E. multilocularis*. Gasser et al. (1989) identified several immunogenic proteins from *E. granulosus* protoscoleces excretory/secretory or deoxycholate solubilized somatic antigens. One of the protein antigens with a molecular mass of 27 kDa specifically identified 95% of sera from dogs infected with *E. granulosus*. However, the use of this antigen was reportedly inconvenient because it was difficult to obtain adequate quantities of the parasite material for large-scale use in a serological test. A recombinant antigen based on the 10P1 clone of *E. granulosus* showed significant specificity in ELISA but not enough sensitivity for practical use (Gasser et al., 1990). In the present study, 1 cycle of routine purification of Emgp-89 yielded approximately 400 μg, which corresponds to the amount necessary to test 80 serum samples. In addition, the purification of Emgp-89 is relatively easy to achieve by using 2 steps of chromatography. Thus, Emgp-89 derived from *E. multilocularis* protoscoleces may be potentially used as a practical tool for serodiagnosis of intestinal infections in dogs.

Recent research has better elucidated the glycoprotein composition of laminated layer and excretory/secretory fraction of *E. multilocularis* metacestode, including the Em2 and Em492 antigens, 2 metacestode antigen fractions that exhibit immunosuppressive or immunomodulatory properties (Gottstein and Hemphill, 2008; Hülsmeier et al., 2002; Walker et al., 2004). Walker et al. (2004) demonstrated an immunologic relationship between Em2 and Em492 antigens and hypothesized that the Em492 antigen is a

degradation product or subfraction of the Em2 antigen. In the present study, lectin-ELISA profiles of Emgp-89 were similar to those of Em492 antigen. The Em2 antigen was present in the carbohydrate-rich fraction with high molecular weight that eluted at void volume on gel filtration chromatography using Superdex 200HR column (Hülsmeier et al., 2002). To compare this elution profiles we performed gel filtration chromatography under the identical condition. Our Emgp-89 protein showed a similar elution profile in gel filtration chromatography under identical chromatography conditions, suggesting that the structural features of Emgp-89 are similar to those of Em2 and/or Em492 antigen. Em2 and Em492 carbohydrate-rich fractions play a key role in host-parasite interplay. These data suggest that large carbohydrate-rich proteins induce a significant host immune response to intestinal infections with protoscoleces and/or young adult worms. Kato et al. (2005) showed the presence of antibodies specific to protoscoleces E/S antigen in sera from dogs infected with *E. multilocularis*, which is consistent with the above mentioned suggestion.

E. multilocularis infection in dogs could be a serious threat to humans because of the close contact between dogs and their owners. Therefore, surveillance is important to evaluate the prevalence of and develop control programs for this infection. Surveillance and control studies were conducted in many endemic areas (Budke et al., 2004; Dyachenko et al., 2008; Nonaka et al., 2009; Torgerson and Craig, 2009; Ziadinov et al., 2008). Presently, the main technique used to assess the prevalence of *Echinococcus* infection in dogs requires fecal samples. Development of an Emgp-89 -based serodiagnosis would be an alternative technique to verify the findings of these studies and to reduce the risk of human AE.

Acknowledgments

The authors would like to thank Professor S. Nogami and Dr. K. Sawashima for providing serum samples. This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) Grant-in-Aid for Young Scientists (B) #19790310 and Scientific research (B) #20380164.

References

Budke, C.M., Jiamin, Q., Craig, P.S., Torgerson, R.P., 2004. Modeling the transmission of *Echinococcus granulosus* and *Echinococcus multilocularis* in dogs for a high endemic region of the Tibetan plateau. International Journal for Parasitology 35, 163–170.

Budke, C.M., Compos-Ponce, M., Qian, W., Torgerson, P.R., 2005. A canine purgatation study and risk factor analysis for echinococcosis in a high endemic region of the Tibetan plateau. Veterinary Parasitology 127, 43–49.

Deplazes, P., Eckert, J., 1996. Diagnosis of the *Echinococcus multilocularis* infection in final hosts. Applied Parasitology 37, 245–252.

Dyachenko, V., Pantchev, N., Gawlowska, S., Vrhovec, M.G., Bauer, C., 2008. *Echinococcus multilocularis* infections in domestic dogs and cats from Germany and other European countries. Veterinary Parasitology 157, 244–253.

Eckert, J., Gemmell, M.A., Meslin, F.-X., Pawlowski, Z.S., 2001. WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern. Paris, WHO/OIE.

Gasser, R.B., Lightowlers, M.W., Rickard, M.D., 1989. Identification of protein components of *Echinococcus granulosus* protoscolex antigens for specific serodiagnosis of *E. Granulosus* infection in dogs. Parasite Immunology 11, 279–291.

Gasser, R.B., Lightowlers, M.W., Rickard, M.D., 1990. A recombinant antigen with potential for serodiagnosis of *Echinococcus granulosus* infection in dogs. International Journal for Parasitology 20, 943–950.

Gottstein, B., Hemphill, A., 2008. *Echinococcus multilocularis*: the parasite-host interplay. Experimental Parasitology 119, 447–452.

Hülsmeier, A.J., Gehrig, P.M., Geyer, R., Sack, R., Gottstein, B., Deplazes, P., Kohler, P., 2002. A major *Echinococcus multilocularis* antigen is a mucin-type glycoprotein. The Journal of Biological Chemistry 277, 5742–5748.

Kamel, M.M., Maher, K.M., Rabia, I., Helmy, A.H., El-Adawi, A.I., Mousa, M.A., Mahgoub, A.M., 2006. A Con A-purified hydatid glycoprotein fraction effectively diagnoses human hydatidosis. Journal of the Egyptian Society of Parasitology 36, 945–958.

Kapel, C.M.O., Torgerson, P.R., Thompson, R.C.A., Deplazes, P., 2006. Reproductive potential of *Echinococcus multilocularis* in experimentally infected foxes, dogs, raccoon dogs and cats. International Journal for Parasitology 36, 79–86.

Kato, N., Nonaka, N., Oku, Y., Kamiya, M., 2005. Modified cellar immune responses in dogs infected with *Echinococcus multilocularis*. Parasitology Research 95, 339–345.

Katoh, Y., Kouguchi, H., Matsumoto, J., Goto, A., Suzuki, T., Oku, Y., Yagi, K., 2008. Characterization of emY162 encoding an immunogenic protein cloned from an adult worm-specific cDNA library of *Echinococcus multilocularis*. Biochimica et Biophysica Acta 1780, 1–6.

Kouguchi, H., Matsumoto, J., Katoh, Y., Oku, Y., Suzuki, T., Yagi, K., 2007. The vaccination potential of EMY162 antigen against *Echinococcus multilocularis* infection. Biochemical and Biophysical Research Communications 363, 915–920.

Kouguchi, H., Matsumoto, J., Katoh, Y., Oku, Y., Suzuki, T., Yagi, K., 2010. *Echinococcus multilocularis*: Two-dimensional Western blotting method for the identification and expression analysis of immunogenic proteins in infected dogs. Experimental Parasitology 124, 238–243.

Matsumoto, J., Yagi, K., Nonaka, N., Oku, Y., Kamiya, M., 1998. Time-course of antibody response in mice against oral infection with eggs of *Echinococcus multilocularis*. Parasitology 116, 463–469.

Matsumoto, J., Yagi, K., 2008. Experimental studies on *Echinococcus multilocularis* in Japan, focusing on biohazardous stages of the parasite. Experimental Parasitology 119, 534–541.

Montagne, L., Toullec, R., Lalles, P., 2000. Calf intestinal mucin: Isolation, partial characterization, and measurement in ileal digesta with an enzyme-linked immunosorbent assay. Journal of Dairy Science 83, 507–517.

Nonaka, N., Iida, M., Yagi, K., Ito, T., Ooi, H.K., Oku, Y., Kamiya, M., 1996. Time course of coproantigen excretion in *Echinococcus multilocularis* infections in foxes and an alternative definitive host, golden hamsters. International Journal for Parasitology 26, 1271–1278.

Nonaka, N., Kamiya, M., Kobayashi, F., Ganzorig, S., Ando, S., Yagi, K., Iwaki, T., Inoue, T., Oku, Y., 2009. *Echinococcus multilocularis* infection in pet dogs in Japan. Vector-Borne and Zoonotic Diseases 9, 201–205.

Rausch, R.L., 1995. Life cycle pattern and geographic distribution of *Echinococcus* species. In: Thompson, R.C.A., Lymbery, A.J. (Eds.), Echinococcus and Hydatid Disease. Walling ford, CAB International, pp. 89–134.

Sakashita, M., Sakai, H., Kohno, H., Ooi, H.K., Oku, Y., Yagi, K., Ito, M., Kamiya, M., 1995. Detection of *Echinococcus multilocularis* coproantigens in experimentally infected dogs using murine monoclonal antibody against adult worms. Japanese Journal of Parasitology 44, 413–420.

Tanabe, T., Ito, H., Sugiyama, K., Kiriyama, S., Morita, T., 2007. Estimation of luminal mucin content in rats by measurement of O-linked oligosaccharide chains and direct ELISA. Bioscience, Biotechnology, and Biochemistry 71, 575–578.

Thompson, R.C.A., Kapel, C.M.O., Hobbs, R.P., Deplazes, P., 2006. Comparative development of *Echinococcus multilocularis* in its definitive hosts. Parasitology 132, 709–716.

Torgerson, P.R., Budke, C.M., 2003. *Echinococcosis* – an international public health challenge. Research in Veterinary Science 74, 191–202.

Torgerson, R.P., Craig, P.S., 2009. Risk assessment of importation of dogs infected with *Echinococcus multilocularis* into the UK. Veterinary Record 165, 366–368.

Wachira, T.M., Macpherson, C.N., Gathuma, J.M., 1990. Hydatid disease in the Turkanadistrict of Kenya, VII: analysis of the infection pressure between definitive and intermediate hosts of *Echinococcus granulosus*. Annals of Tropical Medicine and Parasitology 84, 361–368.

Walker, M., Baz, A., Dematteis, S., Stettler, M., Gottstein, B., Schaller, J., Hemphill, A., 2004. Isolation and characterization of a secretory component of *Echinococcus multilocularis* metacestodes potentially involved in modulating the host-parasite interface. Infection and Immunity 72, 527–536.

Ziadinov, I., Mathis, A., Trachsel, D., Rysmukhambetova, A., Abdyjaparov, T.A., Kuttubaev, O.T., Deplazes, P., Torgerson, P.R., 2008. *Canine echinococcosis* in Kyrgyzstan: using prevalence data adjusted for measurement error to develop transmission dynamics model. International Journal for Parasitology 38, 1179–1190.

Parasitology International 60 (2011) 84–89

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

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

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ARTICLE INFO

Article history:
Received 18 September 2010
Received in revised form 17 November 2010
Accepted 17 November 2010
Available online 25 November 2010

Keywords:
Taeniid cestode eggs
Species-specific oligonucleotide probes
PCR/dot blot assay

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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doi:10.1016/j.parint.2010.11.005

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

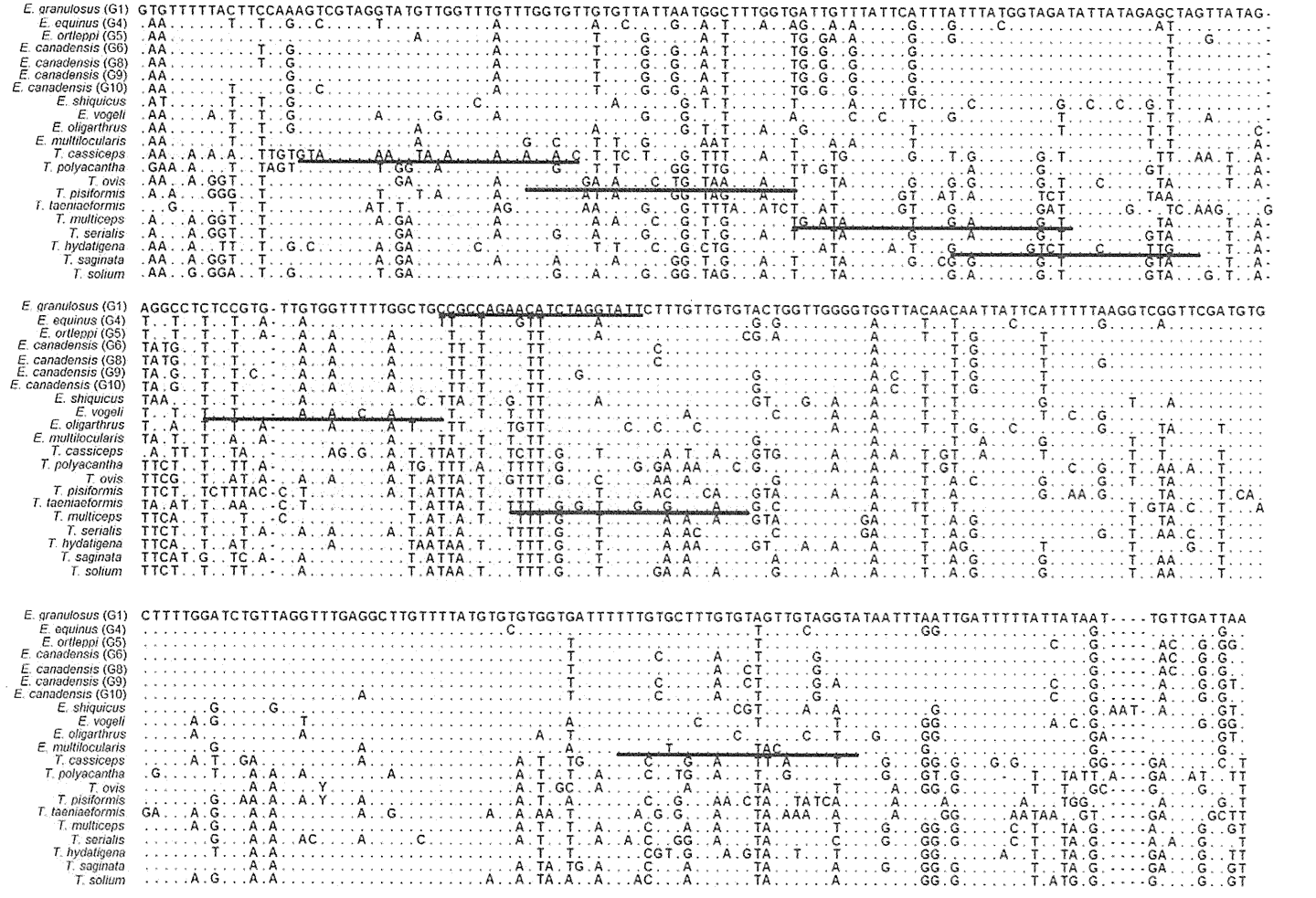

Fig. 1. Multiple alignment of the partial *nad1* sequences containing variable regions in different taeniid species. Sequences were obtained from GenBank. The position of species-specific oligonucleotide probes were underlined and dots represent bases identical to those in the *E. granulosus* genotype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

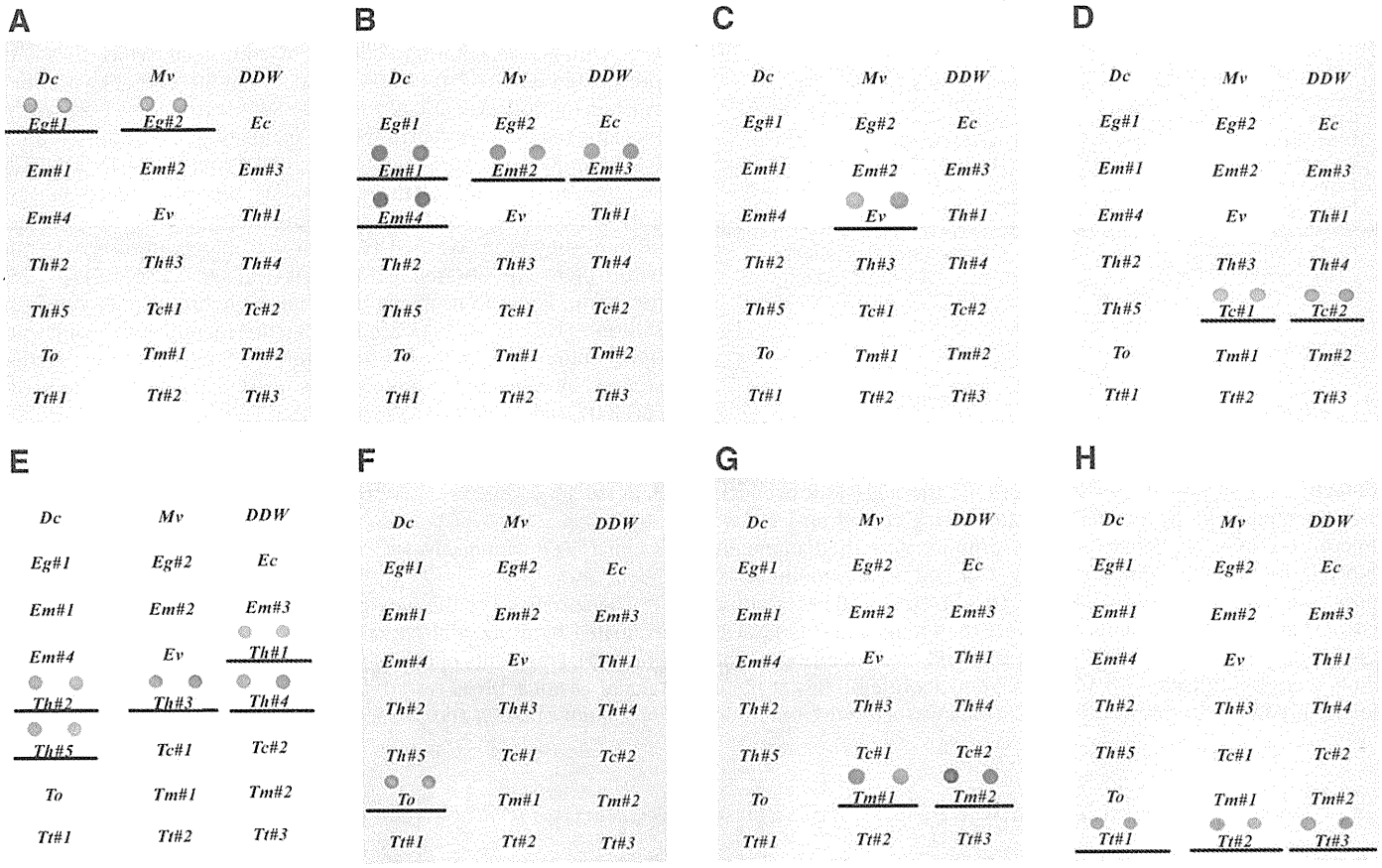

Fig. 2. Dot blot assay performed using adult/larval cestode *nad1* PCR products. Membranes A to H correspond to the *E. granulosus* (genotype 1), *E. multilocularis*, *E. vogeli*, *T. crassiceps*, *T. hydatigena*, *T. ovis*, *T. multiceps* and *T. taeniaeformis* probes, respectively. The PCR products 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

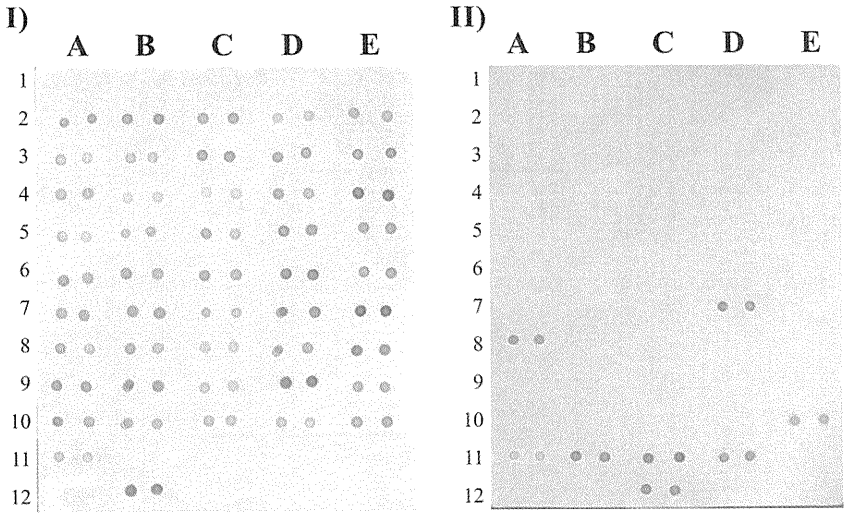

Fig. 3. Dot blot assay of 49 taeniid egg samples. Membrane I) was hybridised with *T. hydatigena* probe, and II) was hybridised with *T. multiceps* probe. As controls, PCR products of *E. granulosus* genotype 1, *E. canadensis* genotype 6, *E. multilocularis* and *E. vogeli* were applied to the first 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.

Acknowledgements

The authors are grateful to Drs. Kentaro Egoshi, Yuka Kato (Hokkaido University), Francis Raoul (University of Franche-Comte), Jean M. Bart (Hôpital J. Minjoz, France) and Perla A. Cabrera (University of the Republic, Uruguay) for providing the parasite samples, and to the members of the Laboratory of Parasitology, Graduate School of Veterinary Medicine, Hokkaido University, for their valuable support. This study was supported by the Japanese Society for the promotion of Science (grant nos. 15380205, 19580353 and 20380164), by the 21st Century COE Program "Program of Excellence for Zoonosis Control", MEXT, and by the Ministry of Health, Labour and Welfare, Japan (Grant for "Studies on risk management based on risk analysis of zoonoses").

References

[1] Eckert J, Schantz PM, Gasser RB, Torgerson PR, Bessonov AS, Movsessian SO, et al. Geographic distribution and prevalence. In: Eckert J, Gemmell MA, Meslin FX, Pawlowski ZS, editors. WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern, World Organization for Animal Health and World Health Organization, Paris, France; 2001. p. 100–43.
[2] El-On J, Shelef I, Cagnano E, Benifla M. *Taenia multiceps*: a rare human cestode infection in Israel. Vet Ital 2008;44:621–31.
[3] Arocker-Mettinger E, Huber-Spitz V, Auer H, Grabner G, Stur M. *Taenia crassiceps* in the anterior chamber of the human eye. A case report. Klin Monatsbl Augenheilkd 1992;201:34–7.
[4] Cabrera PA, Irabedra P, Orlando D, Rista L, Harán G, Viñales G, et al. National prevalence of larval echinococcosis in sheep in slaughtering plants *Ovis aries* as an indicator in control programmes in Uruguay. Acta Trop 2003;85:281–5.
[5] Sissay MM, Uggla A, Waller PJ. Prevalence and seasonal incidence of larval and adult cestode infections of sheep and goats in eastern Ethiopia. Trop Anim Prod 2008;40:387–94.
[6] Scala A, Cancedda GM, Varcacia A, Ligios C, Garippa G, Genchi C. A survey of *Taenia multiceps* coenurosis in Sardinian sheep. Vet Parasitol 2007;143:294–8.
[7] Huang Y, Yang W, Qiu J, Chen X, Yang Y, Qiu D, et al. A modified coproantigen test used for surveillance of *Echinococcus* spp. in Tibetan dogs. Vet Parasitol 2007;149:229–38.
[8] Casaravilla C, Malgor R, Rossi A, Sakai H, Nonaka N, Kamiya M, et al. Production and characterization of monoclonal antibodies against excretory/secretory products of adult *Echinococcus granulosus*, and their implication to coproantigen detection. Parasitol Int 2005;54:43–9.
[9] Dinkel A, Njoroge EM, Zimmermann A, Wälz M, Zeyhle E, Elmahdi IE, et al. A PCR system for detection of species and genotypes of the *Echinococcus granulosus*-complex, with reference to the epidemiological situation in eastern Africa. Int J Parasitol 2004;34:645–53.
[10] Gasser RB, Chilton NB. Characterization of taeniid cestodes species by PCR-RFLP of ITS2 ribosomal DNA. Acta Trop 1995;59:31–40.

[11] Trachsel D, Deplazes P, Mathis A. Identification of taeniid eggs in faeces from carnivores based on multiplex PCR using targets in mitochondrial DNA. Parasitology 2007;134:911–20.
[12] Wu X, Zhang J, Chao L, Liang J, Lu Y, Li H, et al. Identification of rifampin-resistant genotypes in *Mycobacterium tuberculosis* by PCR-reverse dot blot hybridization. Mol Biotechnol 2008;41:1–7.
[13] McElnea CL, Cross GM. Methods of detection of *Chlamydia psittaci* in domesticated and wild birds. Aust Vet J 1999;77:516–21.
[14] Kim A, Choi W, Chung Y, Kim K, Jee Y, Cho H, et al. Utility of RT-PCR-based dot-blot hybridization for detecting and genotyping echoviruses. J Bacteriol Virol 2007;37: 153–60.
[15] Lavikainen A, Haukisalmi V, Lehtinen MJ, Henttonen H, Oksana A, Meri S. A phylogeny of members of the family Taeniidae based on the mitochondrial cox1 and nad1 gene data. Parasitology 2008;135:1457–67.
[16] Gasser RB, Zhu X, McManus DP. NADH dehydrogenase subunit 1 and cytochrome *c* oxidase subunit I sequences compared for members of the genus *Taenia* (Cestoda). Int J Parasitol 1999;29:1965–70.
[17] Nakao M, McManus PD, Schantz PM, Craig PS, Ito A. A molecular phylogeny of genus *Echinococcus* inferred from complete mitochondrial genomes. Parasitology 2007;134:713–22.
[18] Obwaller A, Schineider R, Walochnik J, Gollacknoer B, Deutz A, Janitschke K, et al. *Echinococcus granulosus* strain differentiation based on sequence heterogeneity in mitochondrial genes of cytochrome *c* oxidase-1 and NADH dehydrogenase-1. Parasitology 2004;128:569–75.
[19] Bowles J, McManus DP. NADH dehydrogenase 1 gene sequences compared for species and strains of the genus *Echinococcus*. Int J Parasitol 1993;23:969–72.
[20] Cabrera M, Canova S, Rosenzvit M, Guarnera E. Identification of *Echinococcus granulosus* eggs. Diagn Microbiol Infect Dis 2002;44:29–34.

[Research Note]

Morphological and molecular characterization of sylvatic isolates of *Trichinella* T9 obtained from feral raccoons (*Procyon lotor*)

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The genus *Trichinella* (Trichinellidae: Trichinellidea: Enoplida) has a worldwide distribution in domestic and/or sylvatic animals, and it was once believed to be a monospecific group, but this genus now comprises eight species (*Trichinella spiralis, T. nativa, T. britovi, T. murrelli, T. nelsoni, T. pseudospiralis, T. papuae, T. zimbabwensis*) and three additional genotypic variants (T6, T8, T9) that have yet to be taxonomically defined (Pozio and Zarlenga, 2005). Among them, there are two taxa of *Trichinella* in Japan, namely *Trichinella* T9 from raccoon dogs (*Nyctereutes procyonoides viverrinus* and *N. p. albus*), black bears (*Ursus thibetanus*), brown bears (*U. arctos yesoensis*) and foxes (*Vulpes vulpes schrencki*), and *T. nativa* from a red fox (*V. vulpes*) (Nagano *et al.*, 1999; Kanai *et al.*, 2006). Previous work has shown that raccoons (*Procyon lotor*), which have been introduced from North America since the 1970s, are involved in the sylvatic cycle of *Trichinella* in Japan (Kobayashi *et al.*, 2007). We present here additional morphological and molecular analyses of these sylvatic isolates of *Trichinella* from feral raccoons in Hokkaido, Japan.

In the present study, five *Trichinella* isolates that originated from 648 feral raccoons captured in 2004 and 2005 in Hokkaido, Japan were analyzed. *Trichinella* larvae were examined by an artificial digestion method using tongue muscle (Henriksen, 1978). Briefly, individual tongue samples were digested in 1% pepsin-HCl solution with constant gentle stirring for at least 4 hr at 37°C. After the muscle tissues had been digested, the sediment was allowed to settle and was washed several times. The sediment from the last washing was examined for larvae under a dissection microscope. The larvae were preserved in ethanol for morphological examination and as voucher specimens (Reg. Nos. AS 4324, 5342, 5417, 5498, and 5601) in the Wild Animal Medical Center, Rakuno Gakuen University, Japan. Measurements of cyst size were made from the muscular tissue placed on a glass slide under a dissection microscope. Some of the larvae collected were preserved in TE buffer at –30°C until use, and DNA was extracted from five single larvae according to a previously described method (Bandi *et al.*, 1995; Kanai *et al.*, 2006). These larvae were analyzed separately by a multiplex polymerase chain reaction (PCR) following the method of Zarlenga *et al.* (1999). The five sets of primers listed in Table 1 were used for the multiplex PCR with 10 pmol/μl of each primer. Amplification was carried out using *Taq* polymerase (QIAGEN) with Minicycler™ (MJ Research) under the following conditions: preheating at 94°C for 30 sec, annealing at 58°C for 30 sec, and elongation at 72°C for 1 min for 35 cycles. The PCR amplicon was separated by 2.5% agarose gel electrophoresis and stained with ethidium bromide. For further characterization, the nucleotide sequence of a partial mitochondrial cytochrome oxidase subunit I (COI) was determined. Primers (5'-CAC CCA GAA GTA TAC ATC C-3' and 5'-GTA ATA ATA GGT CTA GGG AGG-3') designed based on sequences of *T. nativa* (accession no. DQ007891) and *Trichinella* T9 (DQ 007898) were used for amplification and nucleotide sequencing. PCR was performed under the following conditions: preheating at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 1 min for 40 cycles. PCR products were purified and directly sequenced using DTCS Quick Start Master Mix (BECKMAN COULTER™) with an automatic sequencer (CEQ™ 8000, BECKMAN COULTER™) according to the manufacturers' instructions. All sequences were aligned using GENETYX-Mac ver. 10.1.4 software.

Table 1. Primer pairs used for multiplex PCR (Zarlenga *et al.*, 1999).

Primer pairs	Sequences
I	5'-GTTCCATGTGAACAGCAGT-3
	5'-CGAAAACATACGACAACTGC-3
II	5'-GCTACATCCTTTTGATCTGTT-3
	5'-AGACACAATATCAACCACAGTACA-3
III	5'-GCGGAAGGATCATTATCGTGTA-3
	5'-TGGATTACAAAGAAAACCATCACT-3
IV	5'-GTGAGCGTAATAAAGGTGCAG-3
	5'-TTCATCACACATCTTCCACTA-3
V	5'-CAATTGAAAACCGCTTAGCGTGTTT-3
	5'-TGATCTGAGGTCGACATTTCC-3

Larval cysts which formed in tongue muscles were spindle-shaped and each cyst included a single coiled larva.

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Table 2. The measurements of *Trichinella* larvae obtained from the masseter of the raccoon AS4324 (in mm, n = 10).

	Max	Min	Mean	SD
Cyst length	0.47	0.3	0.336	0.046
Cyst width	0.26	0.2	0.233	0.018
Body length	1.25	0.65	1.056	0.185
Body width	0.04	0.03	0.036	0.003
Esophagus	1.12	0.46	0.774	0.036
Stichosome	0.9	0.36	0.61	0.01
Rectum	0.06	0.03	0.041	0.156

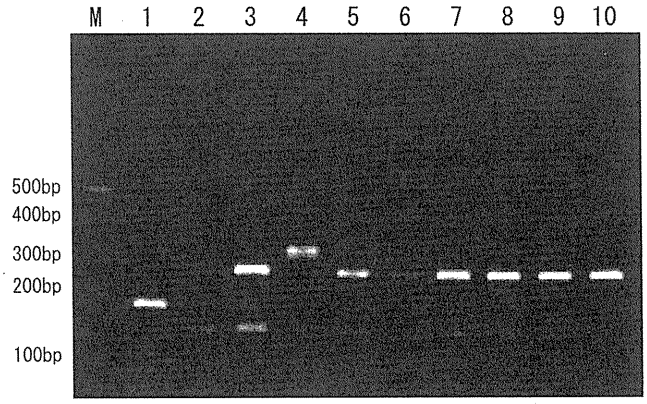

Fig. 1. Electrophoretic pattern after multiplex-PCR amplification of *Trichinella* larvae from feral raccoons and wildlife of Hokkaido Prefecture.
Lane M: 100 bp DNA ladder, lane 1: *T. spiralis* reference larva (code ISS 413), lane 2: *T. nativa* (control, Otofuke fox: Kanai *et al.*, unpublished), lane 3: T9 (control, Sapporo fox: Kanai *et al.* 2007), lane 4: *T. pseudospiralis* (code ISS 13), lane 5: AS5342, lane 6: AS5417, lane 7: AS5498, lane 8: AS4324, lane 9: AS5601, lane 10: T9 (control, Atsuma raccoon dog: Kobayashi *et al.*, unpublished)

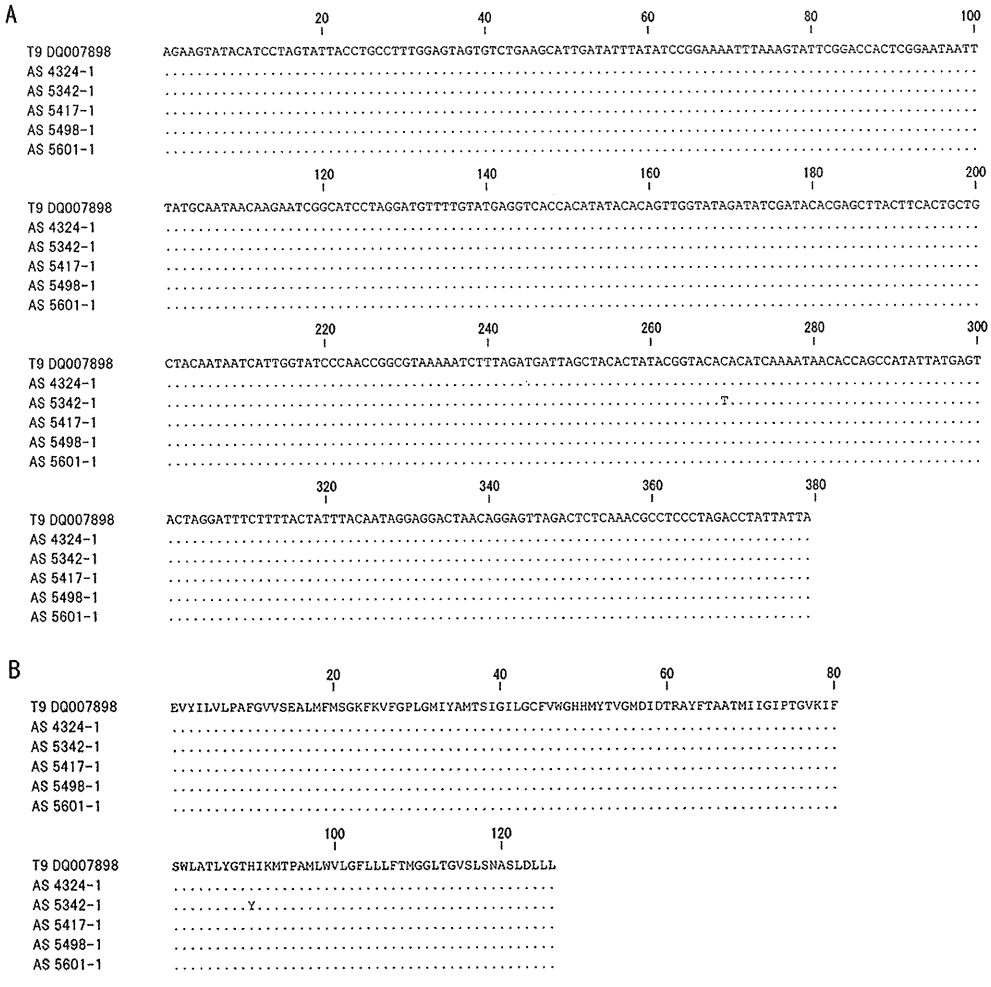

Fig. 1. Alignment of a partial COI sequence of the *Trichinella* larvae.
Nucleotide (A) and amino acid (B) alignments of a partial COI sequence of the *Trichinella* larvae of feral raccoons from Hokkaido with *Trichinella* T9 (accession number DQ007898). Bases that are identical to those of T9 are indicated by dots.

The measurements of the larvae and the cysts are shown in Table 2. Muscle larvae from infected raccoons showed two bands of 127 bp and 253 bp; a pattern specific to *T. britovi* complex (*T. britovi*, T8, T9) (Fig. 1). The nucleotide sequences of a part of the COI gene (379 bp) of larvae from the five raccoons showed highest identities (99.7-100%) to *Trichinella* T9; indeed, the COI sequences of *Trichinella* T9 from five raccoons showed little divergence (Fig. 2). Of the five samples, four (WAMC-AS nos. 4324-1, 5417-1, 5498-1, and 5601-1) had the same sequence (accession number AB 267878), which was completely identical to the previously reported sequences of *Trichinella* T9 from animals of mainland Japan (Nagano *et al.*, 1999). A DNA sequence of *Trichinella* T9 from the remaining sample (WAMC-AS no. 5342-1) showed a single nucleotide polymorphism, which resulted in a single amino acid polymorphism (accession number AB 267879). DNA sequencing of the three other larvae from the same raccoons were analyzed by the same method, and showed the same pattern. *Trichinella* larvae from feral raccoons of Japan were identified by multiplex PCR and COI sequence as *Trichinella* T9.

Raccoons are widely distributed throughout North America, and have also been introduced to Russia and Western Europe. Previously, *T. murrelli* (Pozio and La Rosa, 2000) and *T. psudospiralis* (Garkavi and Gineev, 1976) have been reported in feral raccoons of North America and Russia, respectively. *Trichinella murrelli* is the etiological agent of infection in sylvatic carnivores living in temperate areas of the Nearctic region and *T. psudospiralis* is a cosmopolitan non-encapsulated species infecting both mammals and birds. However, neither *T. murrelli* nor *T. psudospiralis* but T9 has been determined in feral raccoons in Japan. Since *Trichinella* T9 has been reported only in wildlife indigenous to Japan (*i.e.*, a raccoon dog from Yamagata, a black bear from Aomori, and raccoon dogs and foxes from Hokkaido) (Nagano *et al.*, 1999; Kanai *et al.*, 2006), the present results suggested that the raccoons tested here acquired the larvae in the natural ecosystem of Japan.

ACKNOWLEGDEMENTS

The present survey was supported in part by Grant-in-Aid (nos. 14560271, 18510205, S0891002) and by the High Technological Research Center (Rakuno Gakuen Univ.) of The Ministry of the Education, Science and Culture of Japan. We are grateful for the raccoon samples provided to us by the local government offices, EnVision, Hokkaido Forest Management Corporation, Nopporo Natural Forest Park Office, and Raccoon Research Group. We are also grateful to Y. Asakawa, K. Suzuki, G. Abe, and M. Sashika for sampling.

LITERATURE CITED

Bandi, C., La Rosa, G., Bardin, M.G., Damiani, G., Cominicini, S., Tasciotti, L. and Pozio, E. (1995) Random amplified polymorphic DNA fingerprints of the eight taxa of *Trichinella* and their comparison with allozyme analysis. Parasitology 110, 401-407.

Garkavi, A. M. and Gineev, B. L. (1976) *Trichinella* in wild animals in northern Caucasus. Voprosy Prirodnoi Ochagovosti Boleznei 8, 136-139.

Henriksen, S. A. (1978) Recovery of *Trichinella spiralis* larvae from frozen muscle samples. Acta Veterinaria Scandinavica 19, 466-468.

Kanai, Y., Nonaka, N., Katakura, K. and Oku, Y. (2006) *Trichinella nativa* and *Trichinella* T9 in the Hokkaido island, Japan. Parasitology International 55, 313-315.

Kobayashi, T., Kanai, Y., Ono, Y., Matoba, Y., Suzuki, K., Okamoto, M., Taniyama, H., Yagi, K., Oku, Y., Katakura, K. and Asakawa, M. (2007) Epidemiology, histopathology, and muscle distribution of *Trichinella* T9 in feral raccoons (*Procyon lotor*) and wildlife of Japan. Parasitology Research 100, 1287-1291.

Nagano, I., Wu, Z., Matsuo, A., Pozio, E. and Takahashi, Y. (1999) Identification of *Trichinella* isolates by polymerase chain reaction--restriction fragment length polymorphism of the mitochondrial cytochrome c-oxidase subunit I gene. International Journal for Parasitology 29, 1113-1120.

Pozio, E. and Zarlenga, D. S. (2005) Recent advances on the taxonomy, systematics and epidemiology of *Trichinella*. International Journal for Parasitology 35, 1191-1204.

Pozio, E. and La Rosa, G. (2000) *Trichinella murrelli* n. sp: etiological agent of sylvatic trichinellosis in temperate areas of North America. The Journal of Parasitology 86, 134-139.

Zarlenga, D. S., Chute, M. B., Martin, A. and Kapel, C. M. O. (1999) A multiplex PCR for unequivocal differentiation of all encapsulated and non-encapsulated genotypes of *Trichinella*. International Journal for Parasitology 29, 1859-1867.

Received January 15, 2011