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An *Echinococcus multilocularis* coproantigen is a surface glycoprotein with unique *O*-gycosylation

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A major surface constituent of *Echinococcus multilocularis* adult worms, referred to as an EmA9 antigen, was immunoaffinity purified and identified as a high-molecular-weight glycoconjugate. Labeling studies using the monoclonal antibody MAbEmA9 indicated that this antigen undergoes a regulated expression during the development from the larval to the adult parasite. Chemical modification of carbohydrate by periodate oxidation resulted in a reduced reactivity with antigen-specific antibodies. Non-reductive β-elimination of the purified molecule indicated the presence of *O*-linked glycans attached to threonine residues. Carbohydrate compositional analyses indicated the presence of *N*- and *O*-glycans with the ratio of carbohydrate to protein being 1.5:1 (w/w). *N*- and *O*-linked glycans were released by hydrazinolysis and analyzed as 2-aminobenzamide derivatized glycans by mass spectrometry together with HPLC and enzymatic sequencing. Novel linear *O*-linked saccharides with multiple β-HexNAc extensions of reducing end Gal were identified. *N*-Linked glycans were also detected with oligomannose and mono-, bi-, tri- and tetra-antennary-type structures, most of which were found to be core-fucosylated. Taken together, the results indicate that the EmA9 antigen is a glycoprotein located at the outer surface of the adult *E. multilocularis*. The observation that the EmA9 antigen expression is developmentally regulated suggests an involvement of this glycoprotein in the establishment of the parasite in its canine host.

Keywords: cestodes/hydrazinolysis/MALDI-TOF/TOF-MS/parasite worms

Introduction

The cestode *Echinococcus multilocularis* is the causative agent of human alveolar echinococcosis, a severe tumor-like parasitic disease that can be fatal if left untreated (Torgerson et al. 2008). In common with all other tapeworms, the parasite lacks an intestine and is covered by a syncytial tegument that mediates nutrient uptake and protects against the hosts' immune response. The life cycle of *E. multilocularis* comprises sexually mature, intestinal stages in the definitive host (carnivores), a free-living egg and the infective larval stage in the intermediate host (rodents) (Eckert and Deplazes 2004). Humans can be accidentally infected with the parasite by ingestion of eggs containing an oncosphere released with feces of the definitive host. Activated oncospheres penetrate the intestinal mucosa and enter via the blood circulation various host organs, most commonly the liver, where it further develops to the metacestode stage. This metacestode is built up of a multivesicular structure surrounded by an outer laminated layer and an inner germinal epithelium proliferating in an alveolar pattern. A major constituent of the laminated layer, the Em2(G11) antigen, has previously been defined as a mucin-type glycoprotein and suggested to play a major role in protecting the parasite from immune and other host defense reactions (Hülsmeier et al. 2002). Infection of the definitive host is caused by ingestion of metacestodes that develop within the small intestine into the adult worm.

In contrast to the metacestode laminated layer, scarce information is available about the structure and biological role of the tapeworm's adult stage surface constituents. The external surface coat is a highly dynamic structure with a short turnover rate. As has been shown for other helminths species (Hokke and Deelder 2001; Theodoropoulos et al. 2001; Thomas and Harn 2004), the outer membrane glycoconjugates of adult *E. multilocularis* may have crucial functions in host-parasite interactions, such as protecting the parasite by modulating the host's immune response. On the other hand, glycan determinants within surface and secreted glycoconjugates principally serve to initiate the immune responses against helminths in infected hosts (Nyame et al. 2004). Excretory/secretory antigens derived from axenic cultures of *E. multilocularis* pre-adult stages were successfully applied for the detection of specific antibodies in host sera, and antibodies raised against these antigens were used for the development of coproantigen tests (Deplazes et al. 1992; Morishima et al. 1999).

In the present work, we describe the identification of a diagnostically relevant antigen, immunoaffinity purified from *E. multilocularis* tegument preparations. The biochemical nature of this antigen was not understood previously, and here we provide the first description of a purification of this antigen. Biochemical and mass spectrometric characterization revealed the presence of a developmentally regulated, high-molecular-weight glycoprotein, modified by *N*-linked glycosylation and unique *O*-galactosyl saccharides linked to threonine. This work describes a new type of *O*-glycosylation in animals.

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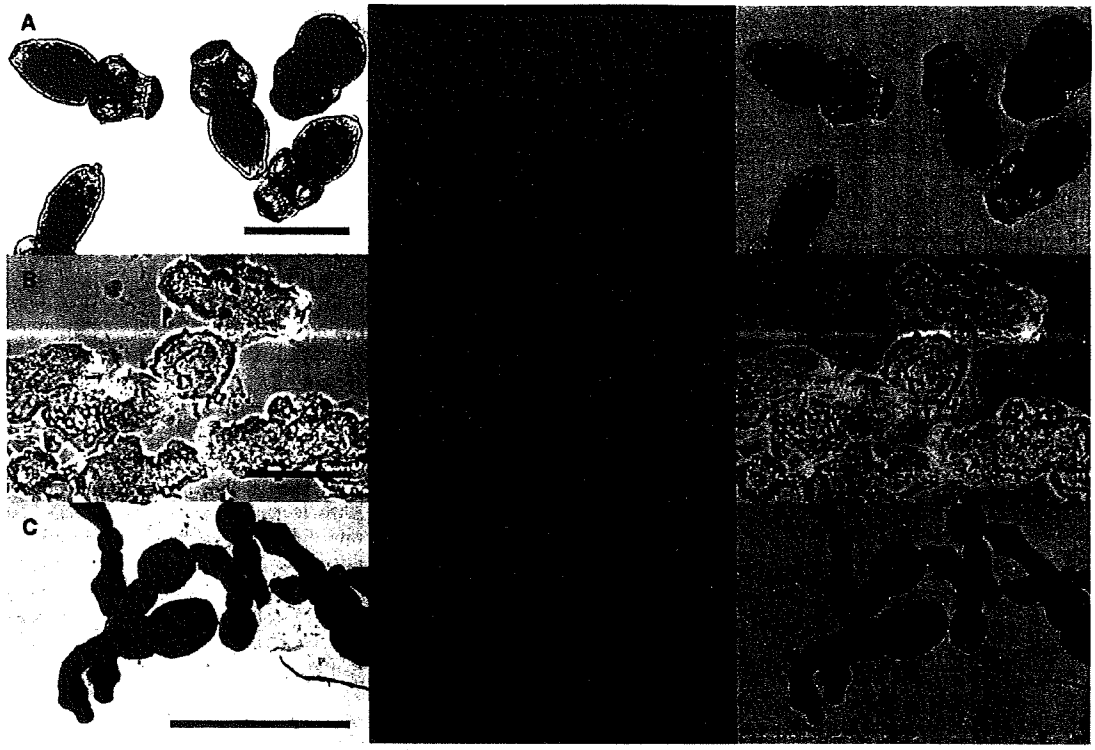

Fig. 1. Immunofluorescence staining of adult and larval stages (protoscoleces) of *E. multilocularis*. The monoclonal antibody EmA9 together with a FITC-conjugated anti-mouse antibody was used for staining. Left column: bright field microscopy. Middle column: immunofluorescence, FITC. Right column: overlay of left and middle column. Row (A): activated protoscoleces. Row (B): freeze fracture section of activated protoscoleces. Scale bar = 100 µm. Row (C): adult worms. Scale bar = 4 mm.

Results

E. multilocularis protoscoleces were isolated from infected *Meriones unguiculatus* voles and labeled with the monoclonal antibody MAbEmA9. Fluorescence detection was mediated by a FITC-conjugated anti-mouse monoclonal antibody. After activating the protoscoleces with acid-bile-pancreatin, a distinct staining of protoscoleces heads could be observed (Figure 1A). Specific antibody binding was not observed in preparations without the monoclonal antibody (conjugate control) or using an unrelated commercial mouse IgG3 antibody as control (data not shown). In freeze fracture sections, a surface location of the corresponding epitope was evident (Figure 1B). Interestingly, in adult *E. multilocularis* an intensive staining of the whole worm was observed (Figure 1C). These data indicate a regulated expression of the *E. multilocularis* EmA9 antigen during development from the larval stages to the adult worm.

The EmA9 antigen was purified from adult worm tegument preparations (Staebler et al. 2006) using an immuno-affinity column prepared with the MAbEmA9. Multiple column eluates were combined and subjected to size-exclusion chromatography. The antigen was detected by ELISA of aliquots from collected fractions. As shown in Figure 2, the antigen eluted from the size-exclusion column as a broad peak, indicating structural heterogeneity of the antigen. The main fractions eluting at 600 kDa were pooled for further analyses.

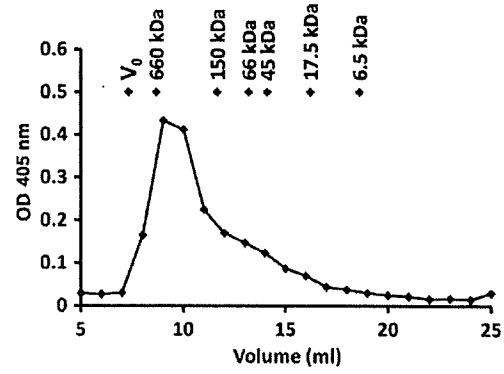

Fig. 2. Size-exclusion chromatography of the EmA9 antigen. One milliliter of fractions was collected and screened by sandwich- ELISA using the monoclonal antibody MAbEmA9. The elution positions of molecular mass standards are indicated with the corresponding molecular weight values.

Crude and purified EmA9 antigen preparations were subjected to sodium periodate oxidation and their reactivities with selected antibodies were assessed by ELISA. Upon oxidation, a clear decrease in signal intensity was observed with both polyclonal chicken anti-tegument antibodies and MAbEmA9,

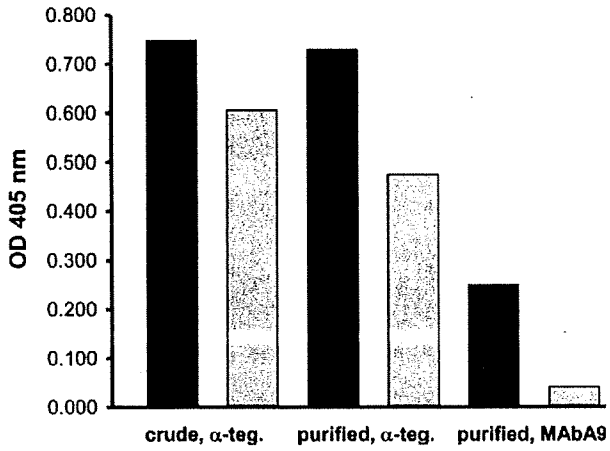

Fig. 3. Sandwich-ELISA analysis of the periodate-oxidized EmA9 antigen. Left: crude *E. multilocularis* tegumental antigen preparation detected with a polyclonal chicken anti-tegument antibody. Middle: the purified EmA9 antigen detected with a polyclonal chicken anti-tegument antibody. Right: the purified EmA9 antigen detected with the monoclonal antibody MAbEmA9. Black bars: without periodate oxidation; gray bars: antigens were subjected to periodate oxidation prior to ELISA analysis. Results are means of duplicate samples.

indicating that carbohydrates constitute major epitopes for these antibodies (Figure 3).

Nonreductive β-elimination of the purified EmA9 antigen resulted in a significant decrease of threonine in the amino acid composition, indicating the presence of *O*-linked glycans attached to the hydroxyamino acid (99.8% confidence, Table I). The amount of serine also appeared to be lowered, suggesting a second *O*-glycosylation type of the antigen. However, with a *p*-value of 0.1648 and a risk level of 0.05, the data do not support a significant decay of serine after β-elimination. Carbohydrate compositional analyses by high-performance liquid chromatography showed that the major carbohydrate constituents of the EmA9 antigen are derived from GlcNAc and Gal, together with significant amounts of Fuc, Man, and GalNAc. The molar ratio of Man:GlcNAc:GalNAc:Gal:Fuc was calculated to be 8:21:1:14:3, and the ratio of carbohydrate to protein was found to be 1.5:1 (w/w).

Glycans were released from the EmA9 antigen by hydrazinolysis under conditions, which release *O*-glycans as well as *N*-glycans (Patel et al. 1993). The liberated glycans were labeled with 2-aminobenzamide (2AB) and analyzed by NP-HPLC. Retention times were calibrated with 2AB-labeled dextran hydrosylate and converted into glucose units (GU) (Grubenmann et al. 2004). An unfractionated, 2AB-labeled sample aliquot was subjected to MALDI-TOF-MS and nine different saccharide compositions could be assigned (S2–S10, Table II). A comparison of fluorescence profiles of glycans released under *N*- and *O*-glycan liberating conditions with *O*-glycan only condition indicated the presence of *N*-glycosylation by the appearance of additional peaks, eluting >5 GU in the *N*- and *O*-glycan releasing condition (data not shown). For subsequent analyses, hydrazinolysis conditions liberating *N*- and *O*-glycans were used (Figure 4). GU values were directly assigned to mass determinations from collected NP-HPLC fractions for the saccharide compositions

Table I. Results of the amino acid and monosaccharide analyses of the EmA9 antigen

Component	Before β-elimination mol%[a]	After β-elimination mol%[a]	*p*-value
Asx	5.4 ± 0.68	4.6 ± 0.32	0.1256
Glx	5.3 ± 0.49	4.7 ± 0.53	0.2030
Ser	4.1 ± 0.07	3.3 ± 0.77	0.1648
His	1.1 ± 0.12	1.0 ± 0.09	0.6371
Gly	4.4 ± 0.63	4.1 ± 0.81	0.6283
Thr	5.6 ± 0.40	3.6 ± 0.28	0.0017
Ala/Arg	14.8 ± 1.33	11.7 ± 1.59	0.0621
Tyr	1.1 ± 0.10	1.0 ± 0.41	0.7001
Val	2.4 ± 0.20	2.4 ± 0.40	0.8924
Met	0.0	0.0	
Phe	1.5 ± 0.05	1.4 ± 0.15	0.6972
Ile	0.0	0.0	
Leu	4.2 ± 0.08	3.7 ± 0.61	0.1948
Lys	n.d.	n.d.	
Pro	2.9 ± 0.18	2.5 ± 0.35	0.1162
Man	7.9 ± 0.88		
GlcN	21.1 ± 1.31		
GalN	1.0 ± 0.20		
Gal	14.2 ± 1.53		
Fuc	2.9 ± 0.24		

[a]The data are background-corrected. Values are means ± SD, $n = 3$, n.d. = not detected. Statistical significance was calculated using the two-tailed Student's *t*-test model with the null hypothesis that the amounts of each amino acid are equal before and after β-elimination. *N*-Acetyl hexosamines were detected as their de-*N*-acetylated derivatives, respectively glucosamine (GlcN) and galactosamine (GalN).

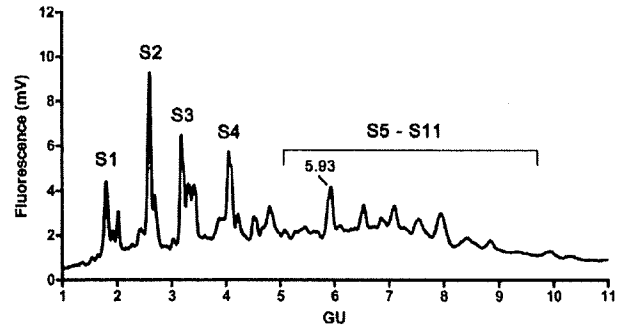

Fig. 4. NP-HPLC analysis of 2AB-labeled EmA9 *O*-linked and *N*-linked oligosaccharides. The retention times were externally calibrated with 2AB-labeled standard glucose oligomers and converted to glucose units (GU). Elution positions of the *O*-glycans S1–S4 and *N*-glycans S5–S11 are labeled. 5.93 GU corresponds to the elution time of a bi-antennary, core fucosylated *N*-glycan.

Hex1HexNAc2, Hex1HexNAc3, and Hex1HexNAc4 with 2.61 GU, 3.18 GU, and 4.06 GU, respectively. From the 2.61 GU and 3.18 GU, fractions collision-induced fragmentation spectra could be obtained by MALDI-TOF/TOF-MS, and the sequences HexNAc-HexNAc-Hex-2AB and HexNAc-HexNAc-HexNAc-Hex-2AB were assigned. Figure 5 shows the fragment spectrum of the saccharide S2 at 2.61 GU. The Y_1-fragment ion at *m/z* 323.1 corresponded to the 2AB-hexose and was also evident in the fragment spectrum corresponding to saccharide S3 (Table II). The Y_2-fragment ion at *m/z* 526.2 was the most

Table II. Summary of the data describing the *O*-linked structures S1-S4 and *N*-glycosylation of the EmA9 antigen

Designation	*m/z* meas.	*m/z* calc.	Δ Mass	Composition	Fragment ions (*m/z*)	JBBH	Reducing end	GU	Structure assignment
S1						+	Gal	1.81	HexNAcβ-Gal
S2	729.280	729.282	0.002	Hex1HexNAc2	226, 244, 305, 323, 351, 429, 447, 508, 526	+	Gal	2.61	HexNAcβ-HexNAcβ-Gal
S3	932.347	932.361	0.014	Hex1HexNAc3	226, 244, 323, 447, 526, 554, 729	+	Gal	3.18	HexNAcβ-HexNAcβ-HexNAcβ-Gal
S4	1135.431	1135.441	0.010	Hex1HexNAc4		+	Gal	4.06	HexNAcβ-HexNAcβ-HexNAcβ-HexNAcβ-Gal
S5	1377.472	1377.493	0.021	Hex5HexNAc2	226, 347, 364, 509, 567, 833, 874, 1036, 1215				Man5
S6	1402.518	1402.525	0.006	dHexHex3HexNAc3	226, 364, 510, 550, 713, 915, 1256				Mono-antennary, core Fuc
S7	1605.589	1605.604	0.015	dHexHex3HexNAc4	364, 510, 538, 567, 713, 741, 915, 931, 1118, 1136, 1402, 1459				Bi-antennary, core Fuc
S8	1751.657	1751.662	0.005	dHex2Hex3HexNAc4	364, 567, 713, 915, 1118, 1264, 1403				Bi-antennary, 1 x Fuc, core Fuc
S9	1767.657	1767.657	0.000	dHexHex4HexNAc4	1264, 1280, 1462, 1621				Bi-antennary, 1 Gal, 1 Fuc
S10	1808.665	1808.684	0.019	dHexHex3HexNAc5	226, 364, 388, 510, 550, 713, 753, 915, 1118, 1321, 1402				Tri-antennary, core Fuc
S11	2012.117	2011.763	0.354	dHexHex3HexNAc6	364, 567, 713, 915, 1321, 1524, 1606, 1809				Tetra-antennary, core Fuc

MALDI-TOF/TOF-MS data: *m/z* meas.: measured values; *m/z* calc.: theoretical values; Δ mass: *m/z* value deviation measured from theoretical values; JBBH: Jack bean beta-*N*-acetyl-hexosaminidase susceptibility, + susceptible to JBBH; reducing end saccharide analysis of JBBH digestion products subjected to RP-HPLC; GU: glucose units determined by NP-HPLC.

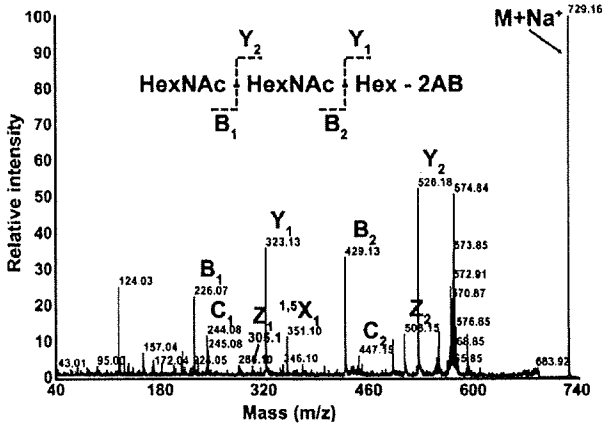

Fig. 5. MALDI-TOF/TOF-MS of the *O*-glycan S2. The fragment spectrum obtained from the peak fractions eluting at 2.61 GU (see Figure 4) together with the assigned saccharide structure is shown. The main fragment ions are labeled; $M+Na^+$: precursor ion.

intense fragment ion in the spectrum and described the disaccharide HexNAc-Hex-2AB. On the other hand, the abundant B_2-fragment ion at *m/z* 492.1 described the HexNAc-HexNAc disaccharide. These two ions can only be generated from a linear structure, suggesting the sequence HexNAc-HexNAc-Hex-2AB for saccharide S2. Apparent Z_1-, Z_2-, C_1-, and C_2-type ions complemented this assignment.

Peak fractions at 1.81 GU, 2.61 GU, 3.18 and 4.06 GU corresponding to S1–S4 were digested with JBBH and rechromatographed by NP-HPLC (Figure 6). Susceptibility to the Jack Bean enzyme could be observed with all four fractions, indicating β-anomericity for the HexNAc constituents and confirming the sequence assignments obtained from MALDI-TOF/TOF-MS. 2AB-labeled chitobiose was treated in parallel with the peak fractions and was completely converted to 2AB-GlcNAc (data not shown). Reverse-phase chromatography of the JBBH-treated fractions showed a co-elution of the reducing end hexose with 2AB-galactose, indicating that galactose is the reducing end constituent in the EmA9 *O*-linked glycans. As shown in Figure 7, 2AB-galactose derived from saccharide S2 was clearly separated from 2AB-mannose, an alternative candidate for the reducing end hexose (compare with Table I).

In unfractionated 2AB-labeled sample aliquots, *m/z* values correlating to possible *N*-glycan structures were also acquired (data not shown). However, after NP-HPLC separation, fragment spectra could not be acquired from the collected peak fractions, thus preventing a direct GU and sequence assignment to the *m/z* values. Therefore, the NP-HPLC fractions

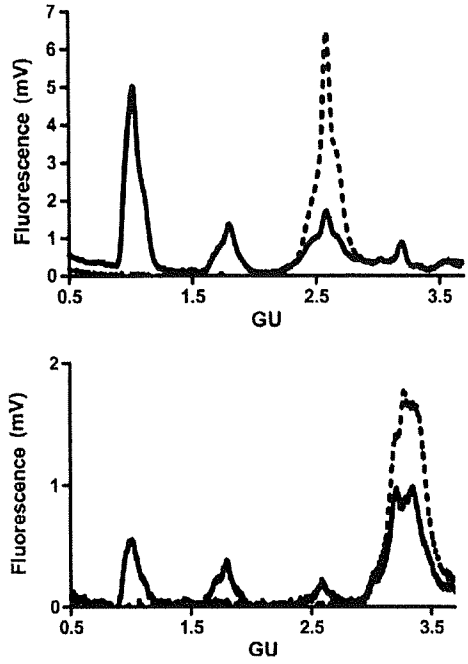

Fig. 6. Jack bean β-*N*-acetylhexosaminidase (JBBH) digestion. NP-HPLC of fractions corresponding to S2 (top panel) and S3 (bottom panel) was re-chromatographed after JBBH digestion (continuous line) or without JBBH digestion (dotted line). Retention times are given in GU (see Figure 4).

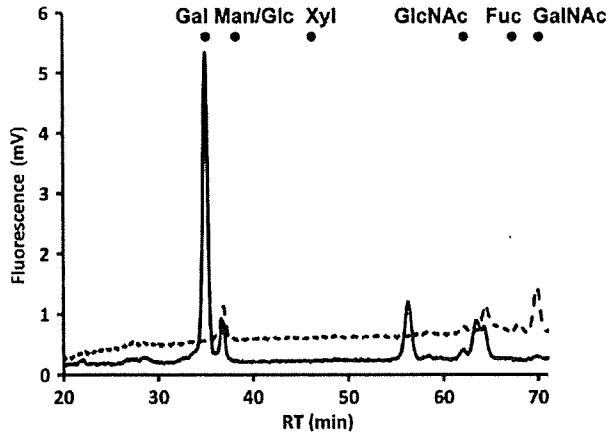

Fig. 7. Reducing-end-saccharide analysis. The NP-HPLC peak fractions corresponding to S2 before (dotted line) and after JBBH digestion were analyzed by RP-HPLC. The elution positions of authentic 2AB-labeled monosaccharides are marked with dots.

corresponding to the elution times of potential *N*-glycans (i.e., >4.2 GU) were pooled, desalted with porous graphitized silica, concentrated and re-analyzed by MALDI-TOF/TOF-MS. Fragment spectra were acquired from oligomannose 5 (Man5) and mono-, bi-, tri- and tetra-antennary-type *N*-glycan structures (Table II). Apart from the Man5 glycan, all *N*-glycans contained

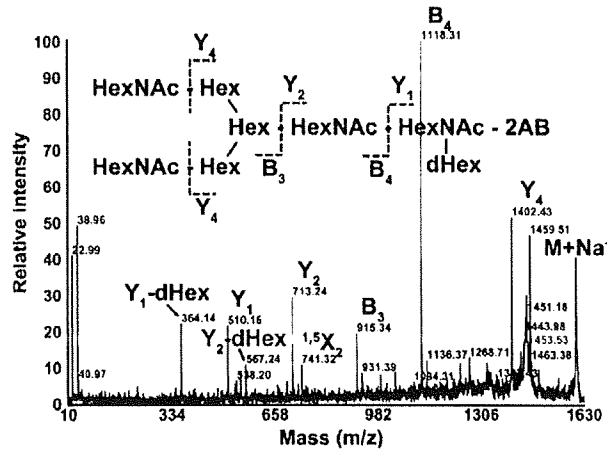

Fig. 8. MALDI-TOF/TOF-MS of the *N*-glycan S7. The fragment spectrum of the precursor ion at *m/z* 1605.589 obtained from the pooled fractions of S5–S11 (see Figure 4) together with the assigned *N*-glycan structure is shown. The main fragment ions are labeled; $M+Na^+$: precursor ion.

one or two fucoses and for all but structure S9, core fucosylation was evidenced by the presence of fragment ions corresponding to dHex-modified Y_1 and/or Y_2 fragments (Figure 8). The second fucose in saccharide S8 might be attached to a terminal HexNAc, as indicated by the presence of a B_4 +dHex fragment ion at *m/z* 1264 (Table II). In addition, fragment spectra from a core fucosylated, tetra-antennary *N*-glycan at *m/z* 2012 could be acquired (S11, Table II). The presence of bisecting *N*-glycans in the acquired spectra cannot be excluded. However, the fluorescence peak at 5.93 GU could be assigned to structure S7 and would be in good agreement with 5.90 and 5.93 GU values described before for bi-antennary core fucosylated *N*-glycans (see Figure 4) (Rudd et al. 1999, 2001).

Discussion

In recent years, a large amount of structural data on helminth-derived glycans has become available due to the application of sensitive and sophisticated analytical techniques primarily HPLC, NMR, and mass spectrometry (Huang et al. 2001). The diverse array of defined oligosaccharide elements includes both *N*- and *O*-glycans of surface and secreted glycoconjugates most of which induce strong antibody responses in parasitized hosts (Khoo and Dell 2001). The glycans in helminth glycoproteins possess structures resembling in many ways those found in higher organisms, but frequently these molecules are specifically modified resulting in a range of novel carbohydrate architectures (Hokke and Deelder 2001). The present study describes the glycan structures of a surface antigen expressed by the adult stages of *E. multilocularis*. This antigen is recognized by the monoclonal antibody EmA9 that has been used as a tool in coproantigen diagnosis of canine echinococcosis (Nonaka et al. 1998). A strong surface expression of the EmA9 antigen could be detected in the adult stages of *E. multilocularis*, whereas only the heads were stained in activated protoscoleces (Figure 1). This is the first observation of the EmA9 epitope in the larval life cycle stage, indicating a developmentally regulated expression

of this antigen. The clear decrease in the immunoreactivity of the EmA9 antigen following periodate oxidation confirmed an association of its antigenic character with glycan structures. Subsequent chemical and statistical analyses showed that the majority of these glycans are O-linked to threonine residues of the peptide backbone. GlcNAc and Gal are the dominant sugar constituents with Gal at the reducing termini. Gal-α-Ser/Thr linkages, extended with one or two alpha-linked Gal, have been reported to occur in cuticle collagens of free living earth- and clamworms (Spiro 2002). The molar percentage of Gly in the amino acid composition of the purified EmA9 antigen speaks against the possibility of a collagen domain in the antigen. Additional peptide sequencing and nucleotide cloning experiments would be required to unravel the identity of the peptide backbone. Further, the anomericity of the Gal to Thr linkage in the EmA9 antigen was not determined. Gal linked in β-anomericity to a peptide backbone has only been described in linkages to hydroxylysine or hydroxyproline (Spiro 2002), and a Gal-β-Thr linkage in the EmA9 antigen would establish a novel type of *O*-glycosylation core structure. Such investigations require large-scale purification of the EmA9 antigen and corresponding glycopeptides followed by enzymatic deglycosylation analyses. *O*-Linked Gal appears generally much less widely dispersed in nature than mucin-type GalNAc-Ser/Thr or the GlcNAc-Ser/Thr linkage. Recently, a single *O*-linked tri-saccharide structure with galactose at the reducing end and elongated with GlcNAc and terminal Gal was described in *Schistosoma mansoni*, whereas the majority of *O*-glycosylation was attributed to mucin-type glycosylation (Jang-Lee et al. 2007). GalNAc-Ser/Thr linkages were not found in the EmA9 antigen, and this glycoprotein differs significantly in its glycoconjugate composition compared to the mucin-type *O*-glycosylation of the Em2(G11) surface glycoprotein expressed by the *E. multilocularis* larval stage. An unusual feature of the EmA9 antigen glycoprotein is the presence of β-HexNAc extensions of reducing end Gal residues. By correlating the high abundance of GlcNAc in the carbohydrate compositional analyses with the major fluorescence peaks of the 2AB-labeled EmA9 antigen saccharides, it can be suggested that β-GlcNAc residues are extending the reducing end Gal residues. To our knowledge, these *O*-linked glycoconjugate structures do not have precedence in any other species described in the literature. *N*-Linked glycans have also been detected with mono-, bi-, tri- and tetra-antennary-type structures, most of which were found to be core-fucosylated and not distinguished by novel structural features. The terminal fucose residue in the *N*-glycan structure S8 might mimik blood group Lewis^a or Lewis^x motives and mediate cellular interactions in the host intestine in support of the manifestation of the parasite infection. Lewis^x epitope bearing glycoconjugates have been described in detergent extracts of the parasitic nematode *Dictyocaulus viviparus* and in antigens derived from parasitic trematodes *Schistosoma* spp. and were implicated in immunomodulatory host responses during establishment of schistosome infection in mice (Velupillai and Harn 1994; Haslam et al. 2000; Nyame et al. 2004).

Previously, a major surface antigen, stage-specifically expressed by the larval stage of *E. multilocularis*, has been characterized as a mucin-type *O*-glycosylated protein (Hülsmeier et al. 2002). The glycans of this Em2(G11) antigen were found to contain novel linear and branched oligosaccharide structures that differ from the adult stage antigen in containing Gal and GalNAc as major constituents with GalNAc being the sole reducing end saccharide. Furthermore, in contrast to the adult stage EmA9 antigen, N-linked glycosylation was not found in the larval stage Em2(G11) antigen (Hülsmeier et al. 2002). Other studies have shown that the adult stages of the dog tapeworm *E. granulosus* also contain epitopes recognized by the MAbEmA9 (Malgor et al. 1997), and indirect methods using lectin binding have identified carbohydrate structures in the tegument and other tissues of this closely related parasite with Gal, GalNAc, and Man as major glycan constituents (Casaravilla et al. 2003). A similar carbohydrate composition pattern appears to be present in other tapeworms including *Taenia taeniaeformis* and *Hymenolepis diminuta*, but the structures of these glycans have also not been defined (Casaravilla et al. 2003). The most extensively and best studied helminth antigenic glycan structures are those carried by schistosomes. These parasites synthesize *O*-glycans ranging from short mucin-type saccharides to highly complex *O*-glycans containing multifucosylated unusual structural elements (Hokke and Deelder 2001). In addition, various stages of this parasite express glycoproteins carrying both, simple and more complex multi-antennary *N*-linked glycans. Many of this great variety of structures have been chemically defined. Interestingly, the glycans of *Echinococcus* and other cestode antigens show some resemblance to the cancer-associated simple *O*-glycosylated antigens (Alvarez Errico et al. 2001). Glycoproteins containing these truncated *O*-glycans, such as the Tn (αGalNAc-*O*-R), TF (βGal1,3-αGalNAc-*O*-R), and Tk [GlcNAcβ1-6(GlcNAcβ1-3)Gal-*O*-R] structures, seem to be widely expressed in helminths (Osinaga 2007; Ubillos et al. 2007).

The precise physiological functions of helminth glycoconjugates are still unclear. Previous studies have shown that immune responses against various helminth species in infected hosts are mainly directed toward glycan structures of surface or secreted glycoconjugates (Ferreira et al. 2000; Nyame et al. 2004). These macromolecules are therefore believed to play critical roles in host-parasite interactions including host recognition, attachment to host tissues, protection of vital tegumental structures, and immune evasion. Like most of the helminth glycoconjugates, the biological role of the EmA9 antigen in the host-parasite interplay is unknown, but the observation that this molecule is developmentally upregulated in the maturing larvae suggests that it plays an important role in the establishment of the parasite in its host and success of the infection. The present study may be a first step toward a better understanding of the interaction between the tapeworm EmA9 antigen and its canine host at the molecular level. The striking differences in glycosylation of the adult versus larval stage antigens reflect an adaptation to the dramatic environmental changes from the definite (canine, small intestine) to the intermediate (rodent, liver) host during the life cycle of *E. multilocularis*.

Material and methods

Immunofluerescence labeling of larval stages and adult worms

Adult stage worms were collected from naturally infected foxes (Deplazes et al. 1999) and conserved with 5% buffered formalin until further processing. *E. multilocularis* larval stages (protoscoleces) were isolated from infected *Meriones unguiculatus* voles and used for immunnofluorescence staining after acid-bile-pancreatin-mediated activation in liquid

preparations or in freeze fracture sections (Thompson et al. 1990). Protoscoleces were stained with and without prior formalin fixation and no differences could be observed in the staining pattern. Formalin-fixed specimens were treated with 50 mM ammonium chloride and 5% fetal calf serum (FCS) in PBS for 20 min and 30 min at room temperature, respectively, and subsequently incubated with the IgG3 monoclonal antibody MAbEmA9 (5 µg/mL PBS) (Kohno et al. 1995) or with 5 µg/mL PBS of an unrelated purified IgG3 MAb (I3784, Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C. The specimens were washed twice with 5% FCS in PBS and incubated with an anti-mouse FITC conjugated secondary antibody (goat anti-mouse IgA, IgG, IgM, F 1010, Sigma), diluted 1:500 in 5% FCS in PBS, for 1 h at 37°C. Then, the specimens were washed once with 5% FCS in PBS and directly examined by fluorescence microscopy (in all experiments conjugate controls without MAbs were performed to confirm specific antibody reactions).

Size-exclusion chromatography

The *E. multilocularis* EmA9 antigen was immunoaffinity purified using the monoclonal antibody MAbEmA9 coupled with CNBr-activated Sepharose. The column was prepared according to the manufacturers' instructions and the eluted antigen was subjected to Superdex 200 HR (GE Healthcare) gel-filtration as described (Hülsmeier et al. 2002).

Amino acid analysis and carbohydrate compositional analyses

Amino acid analysis and nonreductive β-elimination were performed as previously described (Hülsmeier et al. 2002). Changes in amino acid composition were statistically validated using the two-tailed Student's *t*-test model with a risk level of $\alpha = 0.05$ and a degree of freedom calculated as $df = (n_{\text{before}} + n_{\text{after}}) - 2 = 4$.

The *t*-values were calculated with $t = \frac{\bar{X}_{\text{before}} - \bar{X}_{\text{after}}}{\sqrt{\frac{SD^2_{\text{before}}}{n_{\text{before}}} + \frac{SD^2_{\text{after}}}{n_{\text{after}}}}}$.

Carbohydrate analyses were carried out by reversed-phase HPLC of 1-phenyl-3-methyl-5-pyrazolone derivatized monosaccharides (Honda et al. 1989; Fu and O'Neill 1995). Aliquots of purified EmA9 antigen preparation were pre-mixed with 1 nmol lyxose internal standard each. For each batch of samples, two standards of 0.5–1 nmol of mannose (Man), lyxose, *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), glucose, galactose (Gal), xylose, and fucose were prepared. The antigen and standard samples were hydrolyzed in 100 µL of 2 M trifluoroacetic acid (TFA) for 4 h at 100°C in a 0.8 mL crimp top glass vial. The samples were dried in a rotary evaporator and washed with 100 µL of 2-propanol followed by evaporation. Then, 20 µL of 0.5 M 1-phenyl-3-methyl-5-pyrazolone in methanol and 20 µL of 0.3 M NaOH were added and the samples were incubated for 40 min at 70°C. After cooling to room temperature, the samples were neutralized with 10 µL of 0.6 M HCl and vacuum dried to remove methanol. The samples were re-dissolved in 100 µL water and extracted four times with 200 µL dibutyl ether. The upper organic phase was discarded and 10–60% of the sample were analyzed by reversed-phase HPLC using a Vydac 208TP53, 300 Å, C8, 3.2 × 250 mm column, and UV detection at 245 nm. Buffer A was 10% acetonitrile in 50 mM sodium acetate, pH 4.7, buffer B was 25% acetonitrile in 50 mM sodium acetate, pH 4.7, and buffer C was 80% acetonitrile in water.

The following program was used.

Time (min)	% Buffer A	% Buffer B	% Buffer C	Flow (mL/min)
0.0	100			1.0
8.0	100			0.5
10.0	80	20		
48.0		100		
53.0		100		
53.1			100	
56.0			100	
56.1	100			
61.0	100			

Molecular relative response factors versus the lyxose internal standard were calculated for each monosaccharide and the amounts of each monosaccharide in the sample were calculated with respect to the response factor in relation to the lyxose internal standard in the sample.

Periodate oxidation

Aliquots of crude or purified EmA9 antigen fractions were incubated in 50 mM sodium meta-periodate in PBS in the dark for 3 h at room temperature. An equal volume of freshly prepared 1 M sodium borohydride was added, and the reaction mixture was incubated for 1 h at room temperature. The antigen preparations were dialyzed against PBS and subjected to ELISA antigen detection as described (Deplazes et al. 1992).

Hydrazinolysis

The purified EmA9 antigen was extensively dialyzed against 0.1% TFA at 4°C, lyophilized, and subjected to hydrazinolysis for 4 h at 95°C as described (Patel et al. 1993). For re-*N*-acetylation of hexosamines, acetic acid anhydride was added to ice cold, saturated sodium bicarbonate dissolved reaction products. Reducing end acetylations were removed by passage through 3 mL Dowex AG50 resin and incubation in 2 mL of 1 mM copper acetate in 1 mM acetic acid for 1 h at room temperature. Liberated oligosaccharides were purified by passage through 2 mL ODS resin over 1 mL Dowex AG50 and subjected to 2-amino benzamide (2AB) labeling and normal-phase (NP) HPLC analyses as described earlier (Grubenmann et al. 2004).

Mass spectrometry

The MALDI matrix was prepared by suspending 10 mg DHB in 1 mL of 50% acetonitrile, containing 1 mM NaCl. The sample and matrix were mixed on the MALDI plate at a ratio of 1:1 and allowed to dry at room temperature. The dried spots were re-crystallized by applying <0.1 µL ethanol. MALDI mass spectra were recorded in positive ion mode, using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems). Averages of 2000–5000 laser shots were used to obtain MS/MS spectra. The collision energy was set at 1 kV and the air pressure inside the collision cell was set at 2×10^{-6} Torr.

Jack bean β-N-acetylhexosaminidase (JBBH) digestions

Two equal aliquots per pooled peak fractions collected from NP-HPLC were dried by rotary evaporation. One tube was subjected to JBBH (Prozyme) digestion and the other tube was incubated in the digestion buffer only. Enzymatic digestion was carried out

in 20 µL volumes of 0.1 M sodium acetate buffer, pH 5.5, and 10 U JBBH per mL. The digestion mixture was overlaid with 10 µL mineral oil and incubated at 37°C for 16 h. The reaction products were re-analyzed by NP-HPLC.

Reducing end saccharide analysis

The 2AB-saccharide fractions obtained after JBBH digestion were applied to reverse-phase chromatography on a Hypersil ODS column, 4.6 × 150 mm, 3 µm particle size (Thermo Electron Corporation) and eluted using a trinary solvent gradient system at a flow rate of 0.8 mL/min at 40°C. Buffer A was 50 mM sodium acetate, pH 4.7, buffer B was buffer A with 10% acetonitrile, and buffer C was 100% acetonitrile.

The following program was used.

Time (min)	% Buffer A	% Buffer B	% Buffer C
0.0	100		
10.0	100		
70.0	85	15	
80.0	20		80
90.0	20		80
100.0	100		
120.0	100		

The elution positions of authentic 2AB-monosaccharide derivatives were determined prior and post-sample application (Chiba et al. 1997).

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Abbreviations

MAb, monoclonal antibody; HexNAc, *N*-acetylhexosamine; Hex, hexose; Gal, galactose; Man, mannose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; MALDI-TOF/TOF-MS, matrix-assisted laser-desorption/ionisation time-of-flight tandem mass spectrometry; JBBH, Jack bean β-*N*-acetylhexosaminidase; NP-HPLC, normal-phase HPLC; 2AB, 2-amino benzamide; GU, glucose units.

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Research Brief

Echinococcus multilocularis: Two-dimensional Western blotting method for the identification and expression analysis of immunogenic proteins in infected dogs

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ABSTRACT

Domesticated dogs are an important potential source of *Echinococcus multilocularis* infection in humans; therefore, new molecular approaches for the prevention of the parasite infection in dogs need to be developed. Here, we identified and characterized an immunogenic protein of the parasite by using a proteome-based approach. The total protein extracted from protoscoleces was subjected to two-dimensional Western blotting with sera from dogs experimentally infected with *E. multilocularis*. Two protein spots showed major reactivity to the sera from infected dogs. The N-terminal amino acid sequences of these spots were identical to the deduced amino acid sequence of the product of the putative *hsp20* gene. RT-PCR and Western blot analyses revealed that the putative *hsp20* gene and its products were expressed in almost all stages of the parasite life cycle. Furthermore, recombinant hsp20 showed specific reactivity to the sera from infected dogs, suggesting that this molecule may facilitate the development of a practical vaccine.

1. Introduction

Alveolar echinococcosis (AE) in humans is a parasitic zoonosis caused by larval stages of *Echinococcus multilocularis* (Thompson and Lymbery, 1995). This parasite requires two different hosts for the completion of its life cycle. Carnivores such as foxes and dogs act as the definitive hosts and various species of rodents act as the intermediate hosts. The adult worm lives in the small intestine of the definitive hosts and releases infectious eggs in the feces of the final host animal. When eggs are accidentally ingested by humans, each egg releases an oncosphere upon stimulation with intestinal juice (especially bile) in the intestinal lumen, and the oncosphere migrates to major organs via the circulation system. Several or more years after initial parasite infection, the larval stage of the tapeworm forms large cysts, mainly in the liver and lungs, causing organ dysfunction in the patients. Although the prevalence of *E. multilocularis* infection in humans is generally low, AE is a highly lethal disease due to the unlimited proliferation and metastasis of the parasite.

In most of the AE endemic areas, the major definitive host animals of *E. multilocularis* are foxes in wild environments, and dogs are not considered to play an essential role in the natural transmission of the parasite. However, dogs exhibit high susceptibilities to infection with the adult parasites, suggesting that they could be an important source of AE infection in humans if they are accidentally infected because of their intimate relationship. One of the strategies to reduce the risk of human infection is to prevent the production and excretion of infectious eggs. Actually, effective protection against parasite infection based on host-protective antigens has been achieved in experimental studies of cystic hydatid disease caused by another *Echinococcus* species, *E. granulosus*. Vaccination with either recombinant proteins or peptide epitopes of egM4, egM9 and egM123 proteins resulted in highly effective protection against canine infection with *E. granulosus* (Zhang et al., 2003, 2006). Fu et al. identified the potent antigen EgA31 (Fu et al., 1999), and Petavy et al. demonstrated that their oral recombinant vaccine for dogs against *E. granulosus* resulted in a significant reduction in worm burden and a significant suppression of egg production by the adult worms in the definitive hosts (Petavy et al., 2008). These experimental results indicate that the prevention of egg production by the parasite is feasible by vaccination of the definitive hosts.

There is intrinsic interest in characterizing the proteins of *E. multilocularis*, especially those to which definitive hosts respond immunologically, because such proteins have potential importance in the development of vaccines. In this study, experimental infections were performed in a safety facility specially designed for

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the experimental *E. multilocularis* infection of definitive host animals. We successfully identified and characterized immunologically dominant proteins that are specifically recognized by sera from dogs infected with *E. multilocularis*.

2. Materials and methods

2.1. Parasite materials and serum samples

Echinococcus multilocularis (Nemuro strain) was obtained from a dog–cotton rat life cycle maintained at the Hokkaido Institute of Public Health. Protoscoleces were collected from a cotton rat and washed with PBS. Approximately 5×10^5 *E. multilocularis* protoscoleces were used for the experimental infection of each dog. Immature and mature adult worms were collected on days 20 and 40 post-infection from the infected dogs, respectively. The worms were first released from the intestinal contents by soaking in PBS to remove dog intestinal mucus and then rinsed several times in PBS. Serum samples were collected at 40 days after infection from infected dogs (males, 16 months old), and sera were stored individually at −30 °C until use. All experiments were performed in a specially designed safety facility (biosafety level 3) in the Hokkaido Institute of Public Health, Sapporo, Japan.

2.2. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Total protein from the *E. multilocularis* protoscoleces was extracted with Triton X-100 according to the method of Furuya et al. (1989). Total protein from the mature adult worm was extracted by homogenizing the worm in lysis buffer containing 25 mM Tris–HCl, 100 mM NaCl and 0.5% Triton X-100 (pH 7.4). The crude extract was treated with cold acetone and centrifuged; then, the pellet was washed with 80% cold acetone. A total of 120 μg of protein was dissolved in 155 μl of rehydration solution (8 M urea, 2 M thiourea, 4% CHAPS, 20 mM dithiothreitol, 1% carrier ampholyte and 0.002% bromophenol blue). 2D-PAGE was performed with a ZOOM IPG system (Invitrogen) basically according to the manufacturer's instructions. Isoelectric focusing was performed with ZOOM strip (pH 3–10 NL) at a step voltage of 200 V for 20 min, 450 V for 15 min, 750 V for 15 min and 2000 V for 30 min. After equilibration for 15 min in LDS sample buffer containing sample reducing agent (Invitrogen), the strip was applied to SDS–PAGE by using NuPAGE 4–12% Bis–Tris gel (Invitrogen). The separated protein spots were detected with CBB, Pro-Q® Emerald 300 glycoprotein gel kit or Pro-Q® diamond phosphoprotein gel stain, as recommended by the manufacturer.

2.3. Western blotting

After 2D-PAGE, the separated proteins were electroblotted onto a PVDF membrane and blocked by incubating in blocking buffer (PBS containing 10% skim milk and 0.1% Tween 20) for 1 h. The membrane was incubated with serum sample (1:400 dilution in blocking buffer) for 1 h. After washing, the membrane was incubated with anti-dog IgG–alkaline phosphatase (AP) conjugate (1:3000 dilution in blocking buffer). The bound antibodies were detected with a BCIP/NBT immunodetection kit (Perkin-Elmer).

Reactivity between recombinant antigen and sera from infected dogs was performed as follows. Approximately 10 μg of recombinant antigen was blotted onto a PVDF membrane. The recombinant protein was first probed with serum sample at a 1:400 dilution in blocking buffer and secondarily probed with AP-labeled anti-dog IgG (1:3000 dilution in blocking buffer). In this assay, serum samples were preabsorbed in 1 ml blocking buffer containing 20 μl of *Escherichia coli* lysate and 6 μg of recombinant ThioHis tag for 2 h at 37 °C.

2.4. N-Terminal amino acid sequence analysis

Separated proteins were electroblotted onto PVDF membrane according to the method of Hirano and Watanabe (1990). The N-terminal amino acid sequence of the target spot was determined with an automated sequence analyzer (Procise 492cLC, Applied Biosystems).

2.5. Preparation of specific antiserum against protein spot separated on 2D-gel

2D-gel spots were visualized by soaking in 1 M KCl at 4 °C for 2 h. The target spot was carefully cut from the slab gel with a wide-bore pipette tip (1000 μl) and then homogenized with forceps in PBS. Approximately 10 μg of protein were administrated to balb/c mice with Freund's complete adjuvant. Thereafter, two boosters with incomplete adjuvant were given to the animals at 2-week intervals.

2.6. Cloning, expression, and purification of recombinant protein

A DNA template encoding the putative hsp20 was cloned by immunoscreening of a cDNA library constructed from protoscoleces mRNA as described previously (Katoh et al., 2008). Recombinant clones expressing reactive proteins were selected with mouse antiserum prepared as described above. Plaque lift and immunodetection was performed with Protran BA85 membrane (Schlicher & Schull) according to the manufacturer's instructions. For construction of pThioHis-putative hsp20, a DNA fragment was amplified by PCR with the following primer sets: 5′-gga tcc gcg ttc gtg ttg ttg tgc t-3′ with a BamHI site and 5′-ctg cag cac aac gaa tag aac att c-3′ with a PstI site. Subcloning and expression of the recombinant DNAs were performed in *Escherichia coli* as described previously (Kouguchi et al., 2005, 2007). The bacteria were cultured in Lennox broth at 37 °C, and protein expression was induced with 0.5 mM IPTG at 32 °C for 5 h. The recombinant antigen was extracted from bacterial cell lysates as an insoluble fraction. The insoluble pellet was subjected to SDS–PAGE. The band corresponding to the ThioHis-putative hsp20 was cut from the gel and transferred to a PVDF membrane for Western blot analysis.

2.7. Sequence analysis of putative hsp20 gene in genomic DNA

Genomic DNA was extracted from mature adult *E. multilocularis* by using a DNeasy genomic kit (Qiagen). The putative *hsp20* gene in genomic DNA was amplified by PCR (primer set: 5′-gga tcc gcg ttc gtg ttg ttg tgc t-3′ and 5′-ctg cag cac aac gaa tag aac att c-3′) and subcloned with a TA cloning kit (Invitrogen). Both strands of the insert were sequenced with primers recommended by the manufacturer.

2.8. RT-PCR analysis

Total RNA was isolated from protoscoleces, immature and mature adult stage of *E. multilocularis* by using Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was performed by using an RT-PCR kit (Applied Biosystems). Reactions were performed in a total volume of 25 μl containing 250 ng of the total RNA samples. The RT mixtures were incubated at 48 °C for 30 min, and the reaction was terminated by heating at 95 °C for 5 min. Specific primer sets were designed from nucleotide sequences of the putative *hsp20* gene as follows: 5′-aag gac gcc tac gaa gtg g-3′ and 5′-ccg tgg gtt gaa tag cca a-3′. PCR was performed

with 1 μl of cDNA in a total volume of 50 μl. The reaction was performed with a Gene Amp PCR system 9700 (Applied Biosystems) under the following thermal cycling conditions: 5 min at 94 °C, followed by 40 repeats of 30 s at 94 °C, 30 s at 56 °C and 30 s at 72 °C, and finally 7 min at 72 °C.

3. Results and discussion

3.1. 2D-PAGE and Western blot analysis

In this study, we used the proteome-based approach including 2D-PAGE and Western blot (2D-WB) analysis, using sera from dogs with or without infection with *E. multilocularis*. The total proteins from the protoscoleces of *E. multilocularis* were separated on a 2D-gel with a pH gradient from pH 3 to pH 10. Approximately 250 protein spots were visualized by CBB staining (Fig. 1A). These proteins on 2D-gels were transferred onto PVDF membranes and incubated with sera samples from five dogs infected with *E. multilocularis*. The 2D-WB analysis revealed at least 19 spots that were recognized by sera from at least one of the infected dogs, whereas uninfected control sera did not exhibit any clear reaction. Unexpectedly, in an identical experiment performed with lysates obtained from mature adults, we did not detect any clear sera-recognized spots that were common to at least two infected dogs.

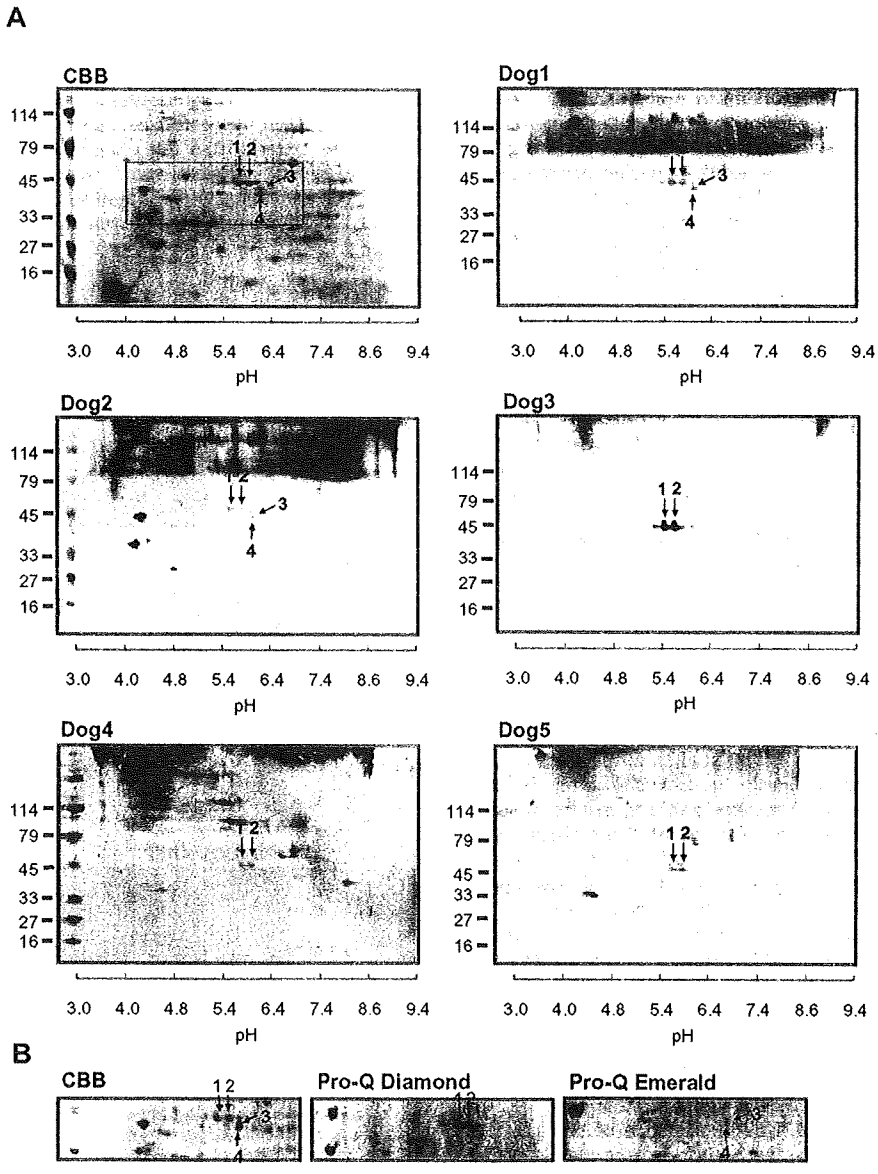

Fig. 1. Identification of immunogenic protein from *E. multilocularis* with 2D Western blot. Total protein was extracted from protoscoleces and 120 μg of the protein was loaded in the first dimension. Molecular size markers are indicated on the left (in kDa). Proteins indicated by spot numbers were analyzed by N-terminal amino acid sequencing. (A) A CBB-stained 2D-gel (CBB) and 2D Western blot using sera from five dogs (dogs 1–5) infected with *E. multilocularis*. (B) Pro-Q® diamond-stained and Pro-Q® Emerald-stained 2D-gel of the boxed area from the CBB-stained gel in (A).

As shown in Fig. 1A (dogs 1–5), two adjacent spots (nos. 1 and 2) located at 43 kDa (p*I* 5.5–6) reacted with all five serum samples. Spot nos. 3 and 4 reacted with two of the five serum samples. The 2D-WBs were overlaid on the corresponding 2D-gel images and the protein spots (nos. 1–4) were analyzed by N-terminal amino acid sequence analysis. The sequence from the spot no. 1 was determined to be MLIFPVRD which were identical to N-terminal of deduced amino acid sequence of putative *hsp20* gene of *E. multilocularis* on database search (protein blast). The sequence of spot no. 2 was also determined as MLIFP identical to that of spot no. 1. As shown in Fig. 1B, these spots were detected by Pro-Q® diamond phosphoprotein staining. Therefore, it was suggested that the two proteins corresponding to adjacent spot nos. 1 and 2 were hsp20 isozymes with different phosphorylation states. This observation is consistent with the fact that the entire amino acid sequence of this molecule contains more than a dozen putative phosphorylation sites (Merckelbach et al., 2003). On the other hand, spot nos. 3 and 4 were detected by Pro-Q® Emerald staining (Fig. 1B), suggesting that these spots corresponding to glycoproteins. The N-terminal amino acid sequences of spot nos. 3 and 4 were determined to be a mixture of two sequences (QJEFTIIM and AGDNLQTE for No. 3, and QPEEIISQ and SLIDDHAF for No. 4). Even when narrow-range pH strips (pH 5.3–6.3) were used, the mixtures in these spots could not be completely separated. A protein blast search revealed no matches for the N-terminal amino acid sequences of these spots. This is an area of that requires further investigations.

In addition, a smear band that reacted with all sera from infected dog was observed at 80 kDa until the top of membrane at a p*I* ranging from approximately 3.5 to 9 (Fig 1A). This high molecular region of the smear band was always detected by 2D-WB using infected dog sera as well as by glycoprotein staining with Pro-Q® Emerald 300 glycoprotein stain kit. No clear reaction was detected with uninfected dog sera. Although a mucin-type major *E. multilocularis* antigen for human patients has been reported by Hülsmeier et al. (2002), no clear reaction was observed between this smear band and sera from human AE patients by 2D-WB in our study (Kouguchi et al., 2008). Therefore we are now attempting to characterize this component as a new antigen candidate for serodiagnosis of the *Echinococcus* infection in dogs.

3.2. Genetic and expression analysis of putative hsp20

Merckelbach et al. (2003) used sera from rabbits immunized with inactivated oncospheres and characterized the putative *hsp20* gene as a clone that expressed immunogenic gene products. However, there have been no further studies on this gene and its products. As mentioned previously, we have shown for the first time that the putative hsp20 protein itself possessed significant reactivity to the sera from dogs infected with *E. multilocularis*. In this study, we successfully cloned the putative *hsp20* gene from a Japanese strain (the Nemuro strain isolated in Hokkaido, Japan) and compared it to the *hsp20* gene in the German strain.

A cDNA expression library was constructed from mRNAs of the larval stage of the parasite (protoscoleces) by using a previously described method (Katoh et al., 2008). Immunoscreening was performed using sera from mice immunized with the putative hsp20 protein obtained from the 2D-gels. A total of 199 positive clones were obtained from approximately 200,000 recombinant phages. Over a 945-bp coding region, the nucleotide sequence of our clone was completely identical to that of the putative *hsp20* gene. We determined the genomic nucleotide sequence of the putative *hsp20* (Genbank Accession No. AB455816): the gene consists of five exons and four introns, and that the lengths of exons 1, 2, 3, 4, and 5 are 183, 150, 198, 124, and 290 bp, respectively. N-Terminal amino acid sequencing revealed that the N-terminal amino acid sequence of the products of putative *hsp20* gene was identical to that of the putative hsp20 protein (MLIFPVRD). This result suggested that our clone and the German isolate have a full-length sequence encoding the putative hsp20, which had not been confirmed to date.

The expression of the putative *hsp20* gene in protoscoleces, immature and mature adult stages was confirmed by RT-PCR. Genomic DNA was included in each assay to assess its contamination, and specific primers were designed to span an intron sequence. In RT-PCR with 40 cycles, the RT-PCR products for the putative *hsp20* gene were detected at the predicted size (325 bp) in protoscoleces, immature and mature adult cDNA samples (Fig. 2A). The specificity of the RT-PCR was confirmed by nucleotide sequencing. No sequence variation was observed in RT products among each stage of the parasite.

Fig. 2B shows the results of 2D-WB analysis of the parasite extract of protoscoleces and mature adult stages with specific antiserum raised against the putative hsp20 protein. As illustrated by CBB staining of the 2D-PAGE gel, the intensity of protein spots corresponding to hsp20 in mature adults was significantly lower than those for hsp20 in the protoscoleces. In contrast, in Western blot images, the extracts from the protoscoleces and mature adults exhibited several adjacent spots with a molecular mass of 43 kDa, corresponding to the putative hsp20 protein. Although the intensity of the hsp20 spots seemed to be different for each

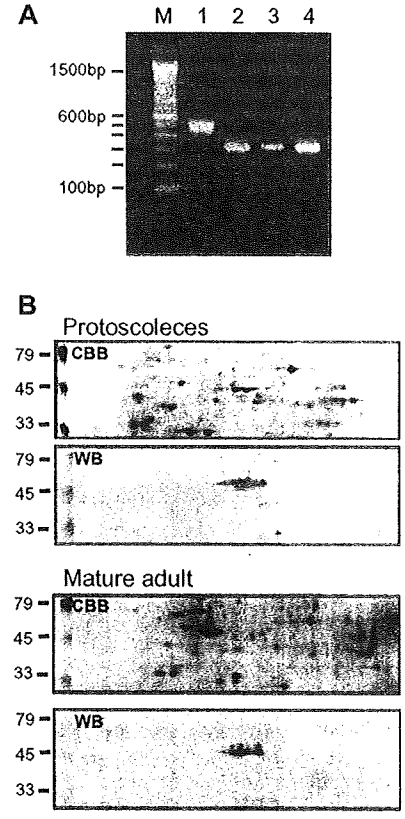

Fig. 2. Expression of putative hsp20 in protoscoleces and adult stages of *E. multilocularis*. (A) RT-PCR analysis for expression of the putative *hsp20* genes in different life-cycle stages of *E. multilocularis*. Total RNAs from protoscoleces, immature adults, and mature adults were reverse transcribed into cDNA and applied to PCR. Molecular size markers are shown in lane M. Lanes 1, 2, 3, and 4 indicate genomic DNA for contamination control, protoscoleces, immature, and mature adult. (B) 2D Western blot of protoscoleces and mature adult samples with anti-putative hsp20. The upper panels show CBB-stained 2D-gel.

stage, the 2D-WB results were consistent with the RT-PCR results, thus showing that the putative hsp20 protein was expressed in the protoscoleces and mature adults of *E. multilocularis*.

Merckelbach et al. (2003) demonstrated that the putative *hsp20* gene is strongly expressed in the oncosphere stage based on the results from a screening experiment with an oncosphere-specific cDNA library of *E. multilocularis*. Taken together with the findings of this study that the gene and protein expression of putative hsp20 were observed in both the protoscoleces and adult stages of the parasite, it is clear that this molecule is expressed in almost all stages of *E. multilocularis* including oncospheres, protoscoleces, and adult worms. Therefore, it appears that the definitive hosts of *E. multilocularis* are continuously exposed to the putative hsp20 produced by the parasite after infection with the protoscoleces. Such a continuous antigenic stimulation with the parasite-derived hsp20 would induce apparent antibody responses to this molecule in the infected animals; these antibody responses create the opportunity to use the putative hsp20 in the vaccine of canine echinococcosis.

3.3. Serological reactivity of the recombinant putative hsp20

Immunological integrity of recombinant hsp20 was confirmed by using sera from infected dogs (10 samples) collected at 40 days after experimental infection and sera from normal dogs as negative controls (10 samples). As shown in Fig. 3, a total of 9 of the 10 sera samples from infected dogs showed a positive reaction to the recombinant antigen, while no significant reaction was observed between the normal sera and the recombinant antigen. These results indicate that the hsp20 protein retained high immunogenicity in infected dogs; this high level of immunogenicity is advantageous for the development of a vaccine antigen to protect *E. multilocularis* infection.

However, despite the presence of anti-hsp20 antibodies in the sera obtained from infected dogs, the parasite persisted until autopsy (40 days after infection). With regard to this aspect, some data are available on the relationship between antibody production in echinococcal infections and the protection conferred by these antibodies. Zhang et al. (2003) found that clones corresponding to a series of genes from the *egM* family (*egM4*, *egM9*, and *egM123*) expressed immunogenic products that reacted with the sera from dogs experimentally infected with *E. granulosus*. Subsequently, they showed that the recombinant antigens, which were created using the *egM* genes, induced a host immune response; this resulted in a significant reduction in the number of worms and in suppression of egg production during *E. granulosus* infection in dogs (Zhang et al., 2006). Recently, a review on the immune response in the definitive host and the protection it offered against echinococcal infection has been published (Zhang et al., 2008); the review indicates that host immunity plays a major role in the natural host–parasite relationship in echinococcosis, and that this immunity is associated with protection against infection.

3.4. Parasite HSPs

HSPs represent dominant antigens in infectious and autoimmune diseases, involving strong cellular immune responses (Zügel and Kaufmann, 1999a). Among the major HSP families, HSP60, HSP70, and HSP90 have been described as major antigens in various infectious diseases caused by nematodes, protozoa, fungi, or bacteria. Furthermore, in various infectious disease models, vaccination strategies that use HSPs have induced significant protection (Zügel and Kaufmann, 1999b). In addition, the unique and potent immunostimulatory properties of some HSPs have been applied to the development of new vaccines in which HSPs act as immunomodulatory/carrier antigens (Mizzen, 1998). However, there are few reports describing HSP members with a small molecular mass of less than 50 kDa (called small HSPs) as antigens during infectious diseases (Norimine et al., 2004; Ferrer et al., 2005; Montalvo-Alvarez et al., 2008).

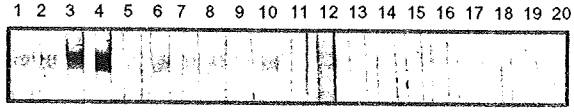

Fig. 3. Serological reactivity of recombinant putative hsp20 to the sera from dogs infected with *E. multiloculars*. Serum samples were diluted 400-fold with blocking buffer. Lanes 1–10 indicate infected dog sera, and normal dog sera (lanes 11–20) were included as a negative control.

As the putative hsp20 of *E. multilocularis* had a molecular weight of 43 kDa, this molecule should be categorized as a small HSP. In this study, we found that the putative hsp20 of the parasite is specifically immunoreactive with the sera of dogs infected with the parasite. In addition, the putative hsp20 was found expressed in different stages, including the protoscoleces and adult worm of the parasite, suggesting that this molecule plays a key role for the parasite development and/or survival in its definitive host animals. Further investigations of the biological function(s) of *E. multilocularis* hsp20 in the host–parasite interplay of the parasite infection would aid in the development of an effective vaccine against canine echinococcosis, as already reported for other parasitic diseases.

Acknowledgments

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SHORT COMMUNICATION

Monitoring of environmental contamination by *Echinococcus multilocularis* in an urban fringe forest park in Hokkaido, Japan

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Abstract
Objectives The aim of this study was to determine the prevalence of *Echinococcus multilocularis* environmental contamination in an urban fringe—the Nopporo forest park of Sapporo city, Hokkaido, Japan. A secondary aim was to determine possible transmission risks areas by associating percentage occurrence of *E. multilocularis*-positive faeces with the different land-use classes.
Methods Wild fox faeces collected from the environment were examined by intravital methods, such as the taeniid egg sucrose floatation technique, *E. multilocularis* coproantigen enzyme-linked immunosorbent analysis and DNA test of taeniid eggs by PCR. Geospatial maps produced by the Global Positioning System and Landsat data were analysed using geographic information system software to determine the association between percentage occurrences of *E. multilocularis*-positive fox faeces and land-use classes.
Results Our findings showed high prevalence rates in both *E. multilocularis* egg and coproantigen-positive faeces (16 and 49%, respectively) in the investigated urban fringe forest park. Data revealed that percentage occurrence of *E. multilocularis*-positive fox faeces was associated with land-use classes, such as forest and open field ($P < 0.05$).
Conclusions We conclude that Nopporo forest park in the urban fringe of Sapporo city, Hokkaido is a reservoir with a high prevalence of zoonotic infective agents for alveolar echinococcosis. Our findings suggest that interface habitats between forests or woodlands and open fields are indispensable for continued maintenance of the life-cycle of *E. multilocularis* and, as such, constitute high risk areas for echinococcosis transmission.

Keywords Environmental contamination · *Echinococcus* eggs · Forest park · Fox faeces

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Introduction

Alveolar echinococcosis, a fatal zoonotic disease caused by the parasite *Echinococcus multilocularis*, is one of the major threats to public health in Japan. To date, there has been a total of 424 reported cases that have been confirmed (surgical method) [1]. The status of most of these patients was considered to be severe and difficult to treat. Although the exact means of transmission is not clear, wild fox populations are widely recognised as the primary source of infective eggs, which are transmitted into the environment through wild fox faeces. Humans and intermediate host

animals can become infected by ingesting *E. multilocularis* eggs present in food and/or water obtained from the contaminated environment.

A dramatic rise in the numbers of urban foxes during the past decades has been documented in Japan and Europe [2–5]. In fact, one study actually anticipated an urban cycle of *E. multilocularis* in Sapporo city [6]. This study recorded an increase in fox populations in urban and suburban areas of Hokkaido, especially protected forest parks and woodlands, and presumed that urban fringe forested areas to be suitable environments for maintaining the life-cycle of *E. multilocularis*. In Zurich, Switzerland, the probable causal factor behind the high prevalence of *E. multilocularis* in foxes in that city was the increased infection pressure among foxes in endemic surrounding areas [7, 8]. Deplazes et al. [3] used various factors to determine the degree of *E. multilocularis* contamination and found that the maximum infection risk areas were villages and urban fringes where rural and urban habitats intersect.

From a public health perspective, monitoring the infection status of wildlife definitive hosts is necessary as part of risk assessment programmes [9]. In the study reported here, we investigated the prevalence of environmental contamination by *E. multilocularis* in a forest park located in the urban fringe of Sapporo city by the intravital diagnosis of fox faeces. We predicted that forest parks and woodlands in urban fringes provide a pool of infective sources for alveolar echinococcosis that represents a significant threat to the highly populated urbanised neighbourhood.

Alternatively, land-use patterns are known to influence rodent population dynamics [10] and, therefore, have important roles in the distribution of *E. multilocularis*. For example, the land-use class grassland has been associated to high alveolar echinococcosis prevalence rates in humans [11, 12]; as such, grasslands are considered to be risk areas in endemic regions in Europe and China. In Hokkaido, however, the Bedford's red-backed vole (*Clethrionomys rufocanus bedfordiae*) is the main prey of foxes [13] and the intermediate host of *E. multilocularis* rather than voles of the genus *Microtus* which is not present in Hokkaido Island. This difference is believed to affect the land-use pattern associated with transmission risk areas in northern Japan. Hence, it may be possible to determine risk areas of transmission to humans through the geospatial mapping of fox faecal locations.

The aim of this study was to determine the prevalence of *E. multilocularis* environmental contamination by an examination of fox faeces. We also determined possible transmission risk areas by associating the percentage occurrence of *E. multilocularis*-positive fox faeces with the different land-use classes.

Materials and methods

The study area was the Nopporo Forest Park (20.5 km^2), which is located about 11–15 km from the centre of Sapporo, the capital city of Hokkaido, Japan (Fig. 1a). The presence of at least two fox families was confirmed in this park. Fox faeces were collected from July to December 2005 on a monthly basis (pooled bimonthly) on forest pathways, roads and agricultural fields located inside and at the peripheries of the park (Fig. 1b). A total of 131 fox faecal samples were collected during the 6-month study period. Prior to the analytical procedures, the faeces were frozen for >1 week at −80°C to render *E. multilocularis* eggs non-infective. Taeniid egg examination was conducted by the centrifugal flotation technique [14] using a sucrose solution with a specific gravity of 1.27. Sandwich enzyme-linked immunosorbent analysis (ELISA) using a monoclonal antibody (EmA9) was used for *E. multilocularis* coproantigen detection [15]. Morphologically, the *Echinococcus* egg is indistinguishable from those of other taeniid species [16]. Taeniid egg-positive faeces were

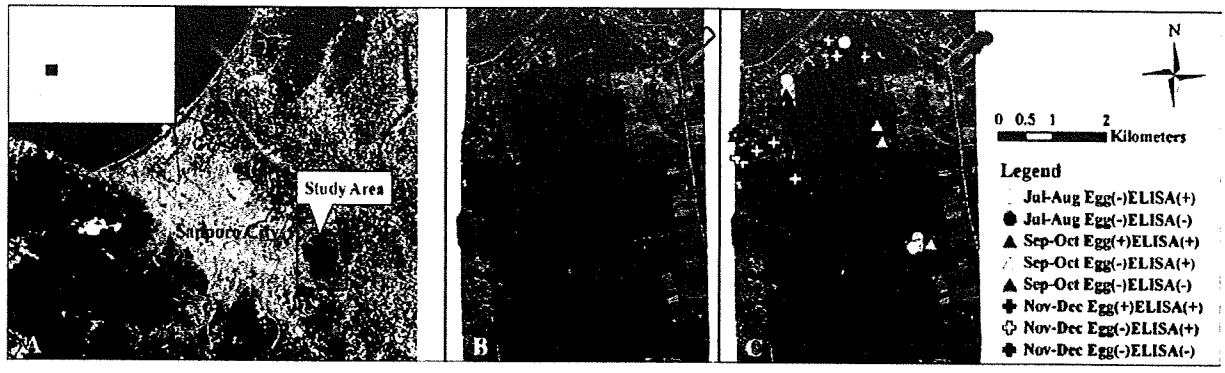

Fig. 1 **a** Map of study area, Nopporo Forest Park, Hokkaido, Japan adjacent to Sapporo city [*inset* is Hokkaido map showing the location below (*rectangle*)]. **b** *Lines* show the roads and pathways investigated. **c** Bi-monthly changes in the distribution of faecal contamination in the environment by foxes infected naturally by *Echinococcus multilocularis*

subjected to copro-DNA examination. Briefly, 1 g of faeces was washed twice with 40 ml distilled water and the sediment resuspended with 30 ml sucrose solution (1.27 g). The supernatant was filtered through a 40-μm nylon mesh and the filtrate subjected to a second filtration through a 20-μm nylon mesh. The sediment was backwashed and centrifuged to obtain the taeniid eggs. DNA was extracted using the QIAamp DNA Mini kit tissue protocol (Qiagen, Valencia, CA). Specific amplification was carried out using the multiplex PCR procedure according to Trachsel et al. [17].

The exact location of fox faeces was recorded (± 50 cm) by a handheld Global Positioning System (GPS; Pathfinder Pro XR; Trimble, Sunnyvale, CA) using ArcPad software 6 (ESRI, Redlands, CA). Data were fed into a personal computer using the Microsoft Active Sync program. Bi-monthly distribution maps of fox faeces were created, and the locations of the faeces were determined using the ArcView 9 software package (ESRI, Redlands, CA). Spatial analysis of the location of fox faeces by land-use classes, such as ground, forest, building, open field, rice field and others, was performed using Landsat data and ArcMap 9 software.

Association between percentage occurrence of *E. multilocularis*-positive faeces and different land-use classes were analysed by the chi-square test. $P < 0.05$ was considered to be significant.

Results and discussion

The results of this study validate an earlier report that urban fringes offer suitable conditions for maintaining the life-cycle of *E. multilocularis* [6]. The prevalence rates of *E. mutilocularis* egg- and coproantigen-positive fox faeces significantly increased on a bi-monthly basis (Fig. 2), with means of 16 and 49%, respectively. Although no

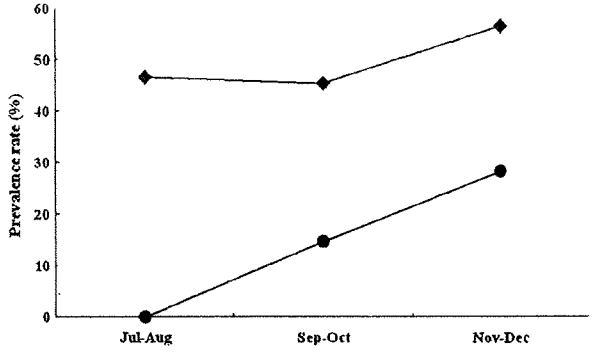

Fig. 2 Prevalence rates of *E. multilocularis* egg- (*circle*) and EmA9 coproantigen (*square*)-positive faeces collected at Nopporo Forest Park at bimonthly intervals

Echinococcus eggs were detected during the period July–August, a prevalence rate of 47% for coproantigen-positive faeces was registered during these months. Coproantigen can be detected during the pre-patent period of infection by this tapeworm before egg excretion is initiated. The prevalence rates of egg- and coproantigen-positive fox faeces in September and October were 14.54 and 45.45%, respectively; these increased remarkably in November and December, reaching 28.26 and 56.52%, respectively. The apparent low egg prevalence compared with the detection of the coproantigen can be accounted for by the intermittent egg excretion of this tapeworm even after maturation [18].

The prevalence rates obtained in our study are comparable to the findings of other surveys carried out in endemic rural areas of Hokkaido [15, 19] and significantly higher than a reported survey performed in an urban setting [6]. Hofer et al. [8] reported that the percentage of *E. multilocularis* infection in the city of Zurich, Switzerland indicated a high prevalence in adjacent areas and that the observed decline in prevalence from recreational to urban areas was due to a lower predation on rodents by urban foxes. It has been reported that high environmental contamination with *E. multilocularis* eggs is a reflection of a high prevalence among definitive hosts, such as foxes [20].

The detection of *E. multilocularis* antigen levels in fox faecal samples collected in the field may provide a pragmatic methodology for the epidemiological surveillance of the infection status in wildlife hosts across large areas as well as provide an indication of the spatial distribution of infected faeces contaminating the environment [9]. The high prevalence rate determined in this study is quite alarming because the park is surrounded by public and residential buildings, schools, sports and camp site facilities. Moreover, it is frequently visited by local tourists, given its location adjacent to Sapporo city (population >1.8 million), the most populous city of Hokkaido.

The copro-DNA test using multiplex PCR [17] identified 21 of 24 egg positive faeces as containing *E. multilocularis* eggs (Fig. 3). Three egg-positive faeces samples showed no reaction during the centrifugal flotation technique with the sucrose solution. None of the samples showed a reaction for *Taenia taeniaeformis* and *E. granulosus*; however, the PCR results suggested that an improved extraction technique for examining *E. multilocularis* copro-DNA should be explored. The absence of eggs despite high coproantigen prevalence rates in July–August may indicate a prepatent or early patent period of infection with a very low level of egg excretion among foxes in the study area. It has been reported that the coproantigen test has a higher likelihood of detecting infection in the late prepatent and early patent phases of infection [21], which is reflected in our results. It has also been reported that in a protected undisturbed population of

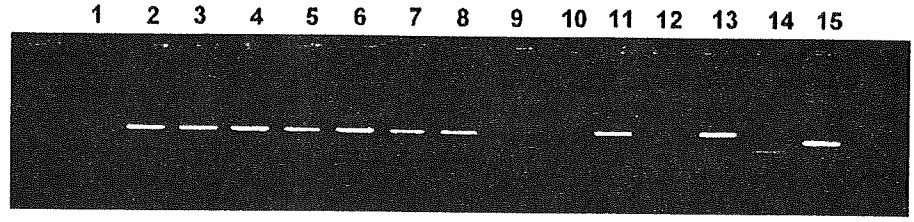

Fig. 3 DNA amplification from ten taeniid-positive fox faeces using multiplex PCR. *Lanes*: *1* 100-bp ladder, *2–11* samples, *12* negative control, *13 E. multilocularis*, *14 E. granulosus*, *15 Taenia taeniaeformis*

foxes in Shiretoku National Park, Japan, infection with *E. multilocularis* ended in the summer, with a new infection being acquired in the early autumn [22]; consequently no eggs were detected during the summer period from the month of August onwards. Similarly, activities of foxes in Nopporo Forest Park were apparently less disturbed.

Geospatial mapping revealed that the locations of fox faeces varied bi-monthly (Fig. 1). Maps showed that fox faeces were mostly distributed at the northeastern part of the park, which happened to be adjacent to an agricultural university with animal and plant research stations. The distribution of fox faeces was also noted in the western area where vegetable gardens were cultivated by local residents. An analysis of land-use classes, however, revealed that the percentage occurrences of *E. multilocularis*-positive fox faeces were significantly higher in forests (33%) and open fields (21%), ($P < 0.05$; Fig. 4). Our data shows that fox faeces were detected more in forests and open fields than near animal stations, plant research plots, vegetable gardens and related agricultural fields. This finding is compatible to the biology of the red-backed voles in Hokkaido, the main prey of red foxes in this area [13], which generally favour both forests and open habitats [23, 24]. While forbs and grasses are the predominate food of *Clethrionomys* during the summer and bamboo grass during the winter, tree bark is also a major food item during the winter [25], thereby necessitating woodlands for their survival. Therefore, an interface between an open habitat and forest is required both in the home range of the red-backed vole and in the maintenance of the *E. multilocularis* life-cycle in Hokkaido. In contrast to Europe and China, the primary habitat of *Microtus* spp. is dependent mainly on grassland. Although infection of intermediate hosts does not require direct contact with definitive hosts, spatial interaction between fox home range and a landscape patch with susceptible small mammals is necessary [11]. Tsukada et al. [6] discovered that ten of the 11 fox den sites detected were located in parks and woodlands in the urban fringes of Sapporo city, a landscape also favourable to voles. In France, the completion of the life-cycle of *E. multilocularis* was found to require a spatial overlap between intermediate host species and definitive host faeces; this study also found that the densities of both *Microtus* sp. and fox faeces were higher in medium-height vegetation edges [26]. It has been suggested that at the regional scale, landscape affects

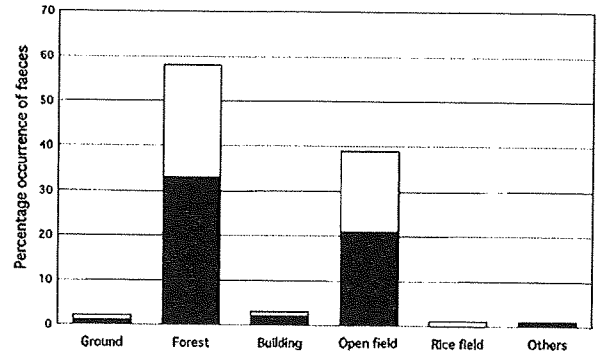

Fig. 4 Percentage occurrence of fox faeces according to land-use classes as analysed using Landsat data. *Filled bar E. multilocularis*-positive faeces, *open bar E. multilocularis*-negative

human disease distribution through an interaction with small mammal communities and their population dynamics [12].

Our data also confirmed that the distribution of fox faeces was associated with the foraging behaviour of the fox and may depend on the availability of food resources in an area [27]. A close relationship between voles and foxes through the parasite *E. multilocularis* in the field has been demonstrated [13].

The deposition of faeces by foxes in open field areas during the summer and fall, which are peak periods associated with soil-linked activities of humans in Hokkaido, increases the transmission risk of *E. multilocularis* eggs. Likewise, agricultural areas were cited as one of the highest factors influencing the urban cycle and infection pressure with *E. multilocularis* eggs [3]. In Europe, the prevalence rates of foxes have risen in many agriculture-dominated areas, particularly in France, The Netherlands, Germany, Austria, Slovakia and Poland; however, the life-cycle of *E. multilocularis* has also been established in many urban areas in which foxes are present at high population densities, thereby presenting an increased infection risk for large human populations [28, 29].

The results of our study confirm that the Nopporo forest park located in the urban fringe of Sapporo city functions as a reservoir, with a high prevalence of zoonotic infective agents for alveolar echinococcosis. We suggest that this interface habitat between forests or woodlands and open

fields is indispensable for the intermediate host voles and for a continued maintenance of the life-cycle of *E. multilocularis*. These environments are contaminated with *Echinococcus* eggs and constitute high transmission risk areas for this fatal infection. Thus, the implementation of a control strategy would be a highly prudent step in these identified high-risk forest parks and woodlands in the urban fringes of Sapporo. A delay in implementing intervention programmes in combination with the increasing number of infected foxes foraging in close vicinity to humans in suburban and urban areas may possibly result in a serious public health hazard.

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