Echinococcus vogeli Infection in a Hunter, French Guiana

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Echinococcus vogeli infection in a hunter from the rain forest of French Guiana was confirmed by imaging and mitochondrial DNA sequence analysis. Serologic examination showed typical patterns for both alveolar and cystic echinococcosis. Polycystic echinococcis caused by *E. vogeli* may be an emerging parasitic disease in Central and South America.

Echinococcosis is one of the most lethal helminthic zoonoses worldwide. The 4 species of the genus *Echinococcus* are *E. granulosus* sensu lato, now including 5 independent species (*1,2*), which causes cystic echinococcosis (CE); *E. multilocularis*, which causes alveolar echinococcosis; *E. vogeli*, which causes polycystic echinococcosis (PE); and *E. oligarthrus*, which causes the recently described unicystic echinococcosis (*3–6*). Among these species, *E. oligarthrus* and *E. vogeli* are neotropical species localized exclusively in Central and South America (*5,6*). Only 3 cases of *E. oligarthrus* infection have been reported in the literature (1 from Brazil, 1 from Venezuela, and 1 from Surinam); 168 *E. vogeli* cases have been reported in 12 countries in Central and South America. To date, there have been no reports of neotropical echinococcosis in Bolivia, Paraguay, Guyana, or French Guiana (*5,6*). *E. granulosus* occurs sympatrically in South America, whereas *E. multilocularis* does not occur there at all (*3,5*). As both *E. vogeli* and *E. oligarthrus* have primarily sylvatic life cycles and the diagnosis is usually based on histopathologic examination of resected lesions, the number of human cases might be underestimated because of the small number of patients who receive surgical treatment (*5,6*). We report a case of human infection from French Guiana caused by *E. vogeli*.

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The Study

In April 2006, a 72-year-old man was admitted to a local hospital in Cayenne, French Guiana, for abdominal pain and a palpable epigastric mass. The patient hunted jaguars in the rain forest of French Guiana and owned dogs. He had no history of travel outside French Guiana. An exploratory laparotomy performed in June 2006 showed a hard, whitish liver tumor, deemed unresectable. Histopathologic examination of a biopsied sample of the tumor showed multilocular cysts. Albendazole treatment was started immediately after surgery. In January 2007, the patient was referred to the Department of General, Endocrine, and Digestive Surgery at the Saint-Louis Hospital, Paris, France. Computed tomography showed a multilocular hypoattenuating cystic mass in the left side of the liver, infiltrating the left glissonian pedicle up to the hepatic hilum (Figure 1, Panel A). Magnetic resonance imaging showed a well-defined, thin-walled, multilocular cystic mass (11 × 10 × 12 cm) involving segments II, III, and IV of the liver (Figure 1, Panel B) and multiple intraperitoneal cysts. The cysts appeared markedly hyperintense on T2-weighted images, hypo-intense on T1-weighted images, and showed slight enhancement of the septa after gadolinium injection. The patient underwent a left hepatectomy and resection of intraperitoneal cysts. Analysis of the operative specimen showed multiple large and small parasite hooks, with mean lengths of 32.7 μm ± 1.6 μm and 42.7 μm ± 0.7 μm, respectively (Figure 2).

Two serum samples from the patient (one obtained in May 2006, the other in December 2006) were analyzed by several immunologic techniques; all indicated infection with *Echinococcus* spp. Commercial ELISAs using *E. granulosus* antigens (Bordier Affinity Products, Lausanne, Switzerland, and Biotrin International, Antony, France) and the *E. multilocularis*-specific Em2^{plus}-ELISA (Bordier Affinity Products, Switzerland) were both strongly positive. Confirmative Western blots (LD Bio Products, Lyon, France) showed a shadow at 16–18 kDa, which is characteristic for *E. granulosus* infection, on both samples. Additional Western blots carried out at Asahikawa Medical College (Asahikawa, Japan) using recombinant antigens (RecAgB8/1, more specific for *E. granulosus*, and RecEm18, more specific for *E. multilocularis*; *7,8*) also showed strong responses. On the basis of serologic patterns obtained with recombinant antigens, with no knowledge of the patient's clinical background, travel history, or images of the lesion, *E. granulosus* infection with many multiple cysts, advanced *E. multilocularis*, or advanced *E. vogeli* infection was suspected (*7,9*).

[1]These authors contributed equally to this article.

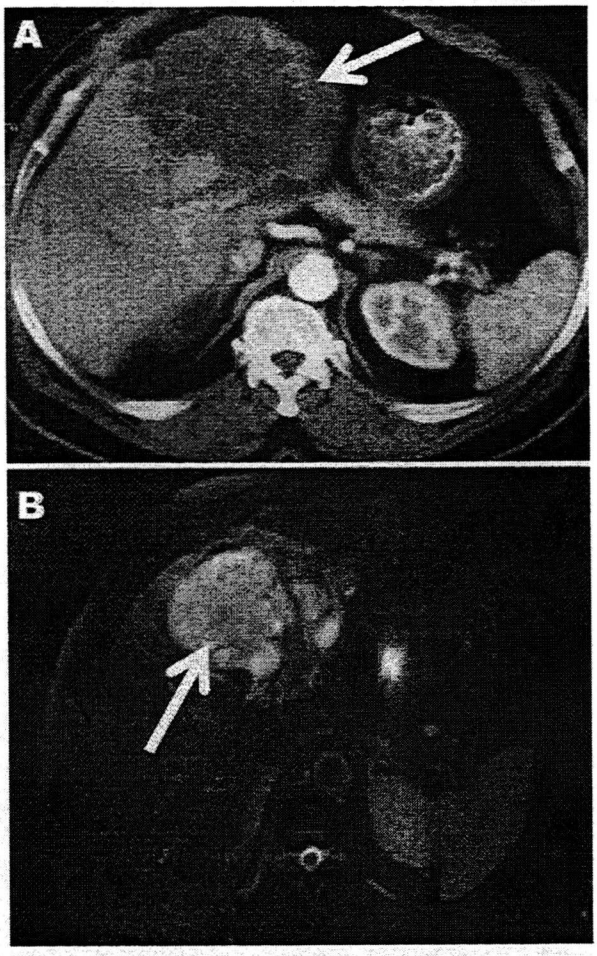

Figure 1. Computed tomography (A) and magnetic resonance (B) images of the liver of a 72-year-old man from French Guiana with polycystic echinococcosis affecting the left side of the liver. White arrows indicate the multicystic liver lesion.

Molecular identification was carried out with reference to the GenBank database by using a highly polymorphic DNA target (*10*). DNA from the liver lesion was extracted by using the High Pure PCR Preparation Kit (Roche, Mannheim, Germany). A part of the *cox1* mitochondrial gene was sequenced by using the *E. vogeli*–specific primer set (*cox1*_F: 5′-TTAATTTTGCCTGGGTTTGG-3′ and *cox1*_R: 5′-ACGACCCATATGATCCCAAA-3′). A sequence of 492 bp was obtained with the ABI 310 sequencer (Applied Biosystems, Foster City, CA, USA) and was compared with *Echinococcus* spp. sequences published in the GenBank database (online Appendix Table, available from www.cdc.gov/EID/content/15/12/2029-appT.htm). The sequences were aligned by using BioEdit 7.0.9.0 (*11*), and sequence identity matrix was generated based on the percentage of base pairs in common between species. The *cox1* sequence was found to be 100% identical to *E. vogeli* species originating from Colombia (GenBank accession no. AB208546; *2*) and was clearly distinguishable from all other *Echinococcus* species (online Appendix Table).

On the basis of imaging showing numerous multiple cysts, serologic examination showing typical patterns for both alveolar and cystic echinococcosis, and the life history of the patient, the diagnosis of polycystic echinococcosis caused by *E. vogeli* could have been made before surgical intervention (*5*,*7*,*9*). The immunoblot showing a strong antibody response to recombinant AgB suggested a large volume of cyst fluid. Therefore, the immunoblot showing strong responses to both recombinant Em18 and AgB may be a typical pattern for advanced *E. vogeli* infection (data not shown). Because few studies using serologic analysis on human *E. vogeli* cases have been published, it would be useful to study antibody responses using recombinant antigens with large numbers of such patients and to compare the results with patterns observed with alveolar and cystic echinococcosis (*7*,*9*).

After surgery, identification of parasite hooks was carried out. The hooks showed the characteristic shape of *E.*

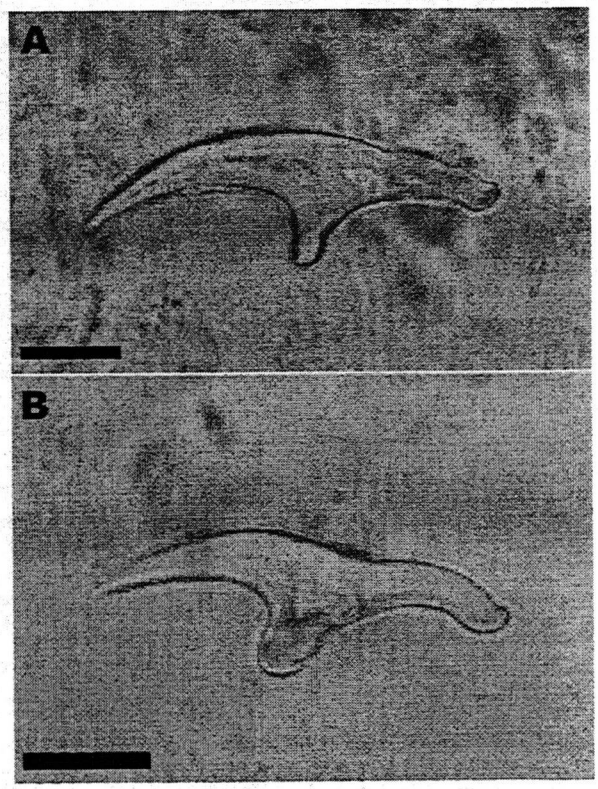

Figure 2. Large (A) and small (B) hooks from *Echinococcus vogeli* protoscoleces in the liver lesion of a 72-year-old man from French Guiana. Scale bars = 10μm.

vogeli and thus differed from *E. granulosus* (mean lengths of large hooks 25.9–35 μm, and small hooks 22.6–27.8 μm) and *E. oligarthrus* (30.5–33.4 μm and 25.4–27.3 μm, respectively) (*12*,*13*). *E. granulosus* and *E. oligarthrus* also occur in South America. The presence of hooks indicated that the parasite lesion was fertile in our patient, as shown in ≈50% of cases (*5*). Based on mitochondrial DNA analysis, the parasite identification was confirmed as *E. vogeli*. Further molecular studies on the haplotypes of this species may give information concerning the genetic diversity and circulation of the parasite in South America (*14*).

Albendazole has been used for medical management of alveolar and advanced cystic echinococcosis (*3*). Several instances of its efficacy on polycystic echinococcosis have been reported, but given the primacy of surgical management of these infections, albendazole will probably remain an additional treatment (*5*).

Neotropical echinococcosis cases are rare compared with alveolar and cystic echinococcosis (*5*). This rarity is probably because of the sylvatic life cycle of these species. However, because domestic dogs have been introduced to areas where *E. vogeli* is present in its natural cycle, the potential for transmission of the parasites from dogs to humans by close contact exists. The at-risk population mainly lives in rural areas and has limited access to medical services, which strongly suggests that many infected persons cannot receive adequate treatment for this underestimated disease.

Conclusions

We report an autochthonous case of *E. vogeli* infection documented in French Guiana. Further investigations are needed to improve the serologic diagnosis of this infection and to define its typical serologic pattern compared with echinococcosis. Healthcare providers need to be alert to the existence of neotropical echinococcosis and should consider the possibility of its emergence in Central and South America. Although rare, this disease is still lethal in untreated cases.

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Geographic pattern of genetic variation in the fox tapeworm *Echinococcus multilocularis*☆

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ABSTRACT

Intraspecific genetic variation of *Echinococcus multilocularis*, the etiologic agent of human alveolar echinococcosis, has been evaluated among 76 geographic isolates from Europe, Asia and North America by using sequence data of mitochondrial and nuclear DNA. Relatively low genetic variation was found only in the mitochondrial DNA sequence consisting of 3 protein-coding genes. Pairwise divergence among the resultant 18 haplotypes ranged from 0.03 to 1.91%. Phylogenetic trees and parsimony network of these haplotypes depicted a geographic division into European, Asian and North American clades, but 1 haplotype from Inner Mongolia was unrelated to other haplotypes. The coexistence of the Asian and North American haplotypes could be seen, particularly on the St. Lawrence Island in the Bering Sea. These data suggest an evolutionary scenario in which distinct parasite populations derived from glacial refugia have been maintained by indigenous host mammals. The nuclear DNA sequence for the immunodominant B cell epitope region of ezrin/radixin/moesin-like protein (*elp*) was extremely conservative, indicating that the *elp* antigen is available for immunodiagnosis in any endemic areas.

1. Introduction

The taeniid cestode *Echinococcus multilocularis* is sporadically distributed throughout the Holarctic ecozone [1]. The endemic areas of central Europe are apparently disconnected from those of Asia, while the Bering Strait is a boundary between North American and Asian endemic areas. The dispersal, isolation and extinction of its mammalian hosts in the Pleistocene (1.8 million to 10 thousand years ago) should be considered to better understand the unique distribution. Repeated glacial events during the Pleistocene probably fragmented and displaced the populations of *E. multilocularis*. The parasite principally utilizes the red fox *Vulpes vulpes* and the arctic fox *Vulpes lagopus* as definitive hosts and many rodents of the families Cricetidae and Muridae as intermediate hosts. The predator–prey relationship of these wildlife perpetuates the parasite's life cycle. Domestic dogs and wolves are also involved as definitive hosts, and the former seems to be an important source for human infection.

Historically, *E. multilocularis* was divided into the geographic subspecies, *E. m. multilocularis* in central Europe, *E. m. sibiricensis* in Alaska and *E. m. kazakhensis* in Kazakhstan [2]. The geographic pattern of genetic variation in *E. multilocularis* was first found in the sequences of mitochondrial DNA (mtDNA) [3,4]. The short mtDNA fragments of cytochrome *c* oxidase subunit 1 (366 nucleotide sites) and NADH dehydrogenase subunit 1 (471 nucleotide sites) were sequenced, and total 4 substitution sites were found between two geographic genotypes named M1 (Europe) and M2 (China, Alaska and North America). It was also reported that the Japanese isolates of *E. multilocularis* belonged to the genotype M2 [5]. Similar classifications into two genotypes were obtained from nuclear genes for 18S rRNA [6] and homeobox [7]. However, the variations detected in the two genes were single nucleotide polymorphisms. The genotyping of microsatellites within U1 snRNA multigene family [8] and anonymous multicopy DNA [9] also showed geographic polymorphisms. These molecular genetic studies are valuable in demonstrating intraspecific variations, but no general conclusion has been made. Phylogenetic studies to clarify the local populations of *E. multilocularis* are still needed to characterize the epidemiological properties of various endemic areas.

In this article, we report the worldwide structure of mtDNA diversity in *E. multilocularis*. Animal mtDNA generally has the following characters: (1) high copy number; (2) non-mendelian maternal inheritance without recombination; (3) homoplasmy in most individuals; and (4) faster evolution than nuclear DNA [10]. These are highly advantageous in assessing genetic diversity at intraspecific level. In this study, a quarter of the whole mtDNA genome was sequenced in each geographic isolate to find informative

☆ Nucleotide sequence data reported in this paper are available in DDBJ/EMBL/GenBank databases under the accession nos. AB461395–AB461420 and AB477009–AB477012.

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substitution sites as many as possible. In addition, a nuclear gene encoding ezrin/radixin/moesin-like protein [11,12] was sequenced to examine the worldwide uniformity of the serodiagnostic antigen. The gene has been demonstrated to be useful for inferring the phylogeny of *Echinococcus* [13].

2. Materials and methods

2.1. Geographic isolates

As shown in Table 1, the geographic isolates of *E. multilocularis* were collected from Austria, France, Germany, Belgium, Slovakia, Kazakhstan, China (Sichuan and Inner Mongolia), Japan (Hokkaido) and United States of America (Alaska, Indiana and South Dakota). The ethanol-preserved adult worms and larval cysts were used as sources for genomic DNA. We considered an adult worm as an isolate because mixed infections with different genotypes possibly occurred in a canid definitive host due to the repeated feeding of infected rodents [14]. Each alveolar cyst from individual rodents and humans was also regarded as an isolate. In total, 76 isolates (44 adult isolates and 32 larval isolates) were examined for this study.

2.2. DNA sequencing

As reported previously [14], adult worms were individually lysed in 10 μl of 0.02 N NaOH at 95 °C for 10 min. DNeasy tissue kit (Qiagen) was used for the purification of genomic DNA from each larval isolate. The adult lysate or the larval DNA was used directly as a template for PCR. PCR was carried out in 50 μl reaction mixture containing 1 μl template, 200 μM of each dNTP, 0.2 μM of each primer, 0.5 U of Ex-Taq polymerase (Takara) and the manufacturer-supplied reaction buffer. Thermal reactions were performed for 35 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 30–90 s).

Primer pairs listed in Table 2 enabled us to amplify the complete mitochondrial genes of cytochrome *b* (*cob*), NADH dehydrogenase subunit 2 (*nad2*) and cytochrome *c* oxidase subunit 1 (*cox1*), and the exons VII, VIII and IX of the nuclear gene encoding the ezrin/radixin/moesin-like protein (*elp*). These *E. multilocularis*-specific primers originated from the sequences of the mitochondrial genome [15] and the *elp* locus [11].

Table 1
Geographic isolates of *E. multilocularis* used in this study.

Regions	Codes [a]	No. isolates [b]	Origins [c]
Europe	AUT	1 (cyst)	Laboratory strain
	FRA	10 (adult)	2 red foxes
	GER	1 (adult)	1 red fox
	BEL	2 (adult)	1 red fox
	SVK	10 (adult)	2 red foxes
Asia	KAZ	5 (cyst)	5 gerbils
	JPN	5 (adult)	1 red fox
		1 (cyst)	1 vole
	CHN-S	10 (adult)	2 dogs
		5 (cyst)	5 humans
		7 (cyst)	7 voles
	CHN-IM	2 (cyst)	Laboratory strain
North America	AK	11 (cyst)	11 voles
	IN	5 (adult)	1 red fox
	SD	1 (adult)	Laboratory strain
Total		76	

[a] AUT, Austria; FRA, France; GER, Germany; BEL, Belgium; SVK, Slovakia; KAZ, Kazakhstan; JPN, Japan (Hokkaido); CHN-S, China (Sichuan); CHN-IM, China (Inner Mongolia); AK, Alaska (St. Lawrence Island); IN, Indiana; SD, South Dakota.
[b] Developmental stages of the parasite are shown in parentheses.
[c] The laboratory strains of *E. multilocularis* were isolated from the local hosts in each locality.

Table 2
PCR primer pairs used for this study.

Targets	Primer pairs (5′-3′) [a]
cob	F: GTTTAAACTGGTAGATTGTGGTTC
	R: CTCCACAGTAGAAATCACCATCA
nad2	F: GCGTTGATTCATTGATACATTGT
	R: TAGTAAAGCTCAAACCGAGTTCT
cox1	F: GACTTTCTCTTTGGTTGGTGTAAG
	R: AACCTAAACAACCAACTTCACAG
elp-exons VII and VIII	F: GACTAAGTTTCACTAAGCTCTA
	R: GCTTCCAAGCTAAATCTGCGTAC
elp-exon IX	F: TTGCATCAATGAATCGGTATTA
	R: CCGCTCTCGAATACTTTAATGGC

[a] F, forward; R, reverse.

The cycle sequencing kits, DYEnamic ET terminator (GE Healthcare) and BigDye terminator (Applied Biosystems), were used for the direct sequencing of PCR products. The resultant sequence ladders were read by ABI PRISM 377 genetic analyzer (Applied Biosystems). Large DNA templates were sequenced by primer walking.

2.3. Computer data processing

In each of the geographic isolates, the nucleotide sequences of the mitochondrial *cob* (1068 sites), *nad2* (882 sites) and *cox1* (1608 sites) were concatenated into a total sequence (3558 sites). The nucleotide sequences of the exons VII (164 sites), VIII (235 sites) and IX (193 sites) in the nuclear *elp* were also combined (592 sites) to compare with the corresponding cDNA sequences of *E. multilocularis* and *E. granulosus*. Multiple alignment files in NEXUS and PHYLIP formats were made manually by editing the plain text files of nucleotide sequences. The software TCS 1.2 [16] was employed to identify individual haplotypes from the alignments. Amino acid sequences were inferred from the nucleotide sequences by echinoderm mitochondrial genetic code [17] or standard genetic code.

The integrated software PAUP 4.0b10 [18] was employed to construct phylogenetic trees by neighbor-joining (NJ) and maximum likelihood (ML) methods. In the tree construction, *Echinococcus shiquicus* was used as an outgroup because this species is a sister taxon of *E. multilocularis* [19]. The NJ tree was constructed from Kimura's two-parameter distances [20] with a gamma shape parameter ($\alpha = 0.5$). The best-fit nucleotide substitution model (TrN + G) was determined for the ML analysis by Akaike Information Criterion (AIC) implemented in MODELTEST 3.7 [21]. Full heuristic search was used to estimate the ML tree. The robustness of phylogenetic trees was tested by bootstrapping with 1000 replicates.

Partitioned Bayesian analysis was performed by using MrBayes 3.1 [22]. The concatenated mtDNA data were divided into 3 partitions corresponding to *cob*, *nad2* and *cox1*. The suitable nucleotide substitution models for *cob* (GTR + I), *nad2* (HKY + I) and *cox1* (GTR + G) were selected by AIC implemented in MrModeltest 2.2 (the modified version of MODELTEST). Using these models, a Metropolis-coupled Markov chain Monte Carlo analysis was run for 1 million generations to estimate the posterior probabilities of the trees. The run produced 10,000 trees, of which 1000 trees were treated as burn-in. The average standard deviation of split frequencies finally reached to 0.01.

A network of mtDNA haplotypes was illustrated by TCS 1.2 [16] using statistical parsimony [23]. The network estimation was run at 95% connection limit. Haplotype frequencies in the network were not used for this study.

Genetic distance between two subpopulations was described by pairwise fixation index (Fst), which was calculated by the software Arlequin 3.1 [24]. The data of mtDNA haplotypes from 6 subpopulations (Europe, China, Alaska, Kazakhstan, Japan and North America) were used for the calculation. Fst values nearing 1 indicate extreme genetic differentiation between two subpopulations.

3. Results

3.1. Characterization of mtDNA haplotypes

As shown in Fig. 1, 18 haplotypes were detected in 76 geographic isolates examined. These were designated as E1 to E5 (European haplotypes), A1 to A10 (Asian haplotypes), N1 and N2 (North American haplotypes) and O1 (other haplotype). When compared with individual genes, the numbers of haplotypes were decreased to 9 in *cob*, 9 in *nad2* and 12 in *cox1*. The concatenation of the genes was effective in detecting haplotypes.

The nucleotide sequence of *nad2* showed the highest frequency of substitution (3.7%, 33 substitution sites/882 sites), followed by *cob* (3.4%, 36/1068) and *cox1* (2.7%, 43/1608). Out of the total 112 substitution sites, 90 sites (80.4%) were transitional changes, and 73 sites (65.2%) were the third codon positions (Fig. 1). Nonsynonymous substitutions were observed in 32 sites (28.6%). In total, 30 parsimony informative sites and 82 singleton sites were found from 18 haplotypes.

3.2. Phylogenetic trees of mtDNA haplotypes

A distance-based NJ phylogram clearly showed that the mtDNA haplotypes were divided into European, Asian and North American clades (Fig. 2). Interestingly, the North American haplotype N1 and the Asian haplotypes A2 and A4 were found to coexist in the St. Lawrence Island in Alaska. In this phylogram, the three geographic clades were supported with high bootstrap values. The North American haplotypes N1 and N2 were deeply branched, but the bootstrap value of their node was not so high. The basal position of the tree was occupied by the haplotype O1 from Inner Mongolia. The maximum value of pairwise divergence reached to 1.91%, when compared between the haplotypes O1 and E1. The divergence values between the European and Asian clades ranged from 0.37 to 0.60%, whereas the values between the Asian and North American clades were from 0.69 to 0.86%. Within the North American clade, the divergence between the haplotypes N1 and N2 was 0.75%.

Rooted cladograms were inferred by ML and Bayesian methods (Fig. 3). Both methods depicted trees with similar topology, showing three geographic clades. The trees demonstrated the monophyly of European and Asian haplotypes, but a branching node into European and Asian clades was not highly supported by bootstrap and Bayesian statistics. The basal node was unresolved in both trees. The haplotype O1 from Inner Mongolia was situated at the basal lineage together with the North American haplotypes N1 and N2.

3.3. Parsimony network of mtDNA haplotypes

The statistical parsimony network of the mtDNA haplotypes was illustrated in Fig. 4. Only the haplotype O1 from Inner Mongolia was disconnected with the network. Among the participants in the network, the Asian haplotype A5 showed the biggest outgroup probability (0.182). The European clade was closely related with the Asian clade. The mutational steps within each geographic clade were infrequent, and their maximum numbers were 6 in the European clade and 10 in the Asian clade. The North American haplotypes N1 and N2 were located far from the European and Asian clades. The network distance between the haplotypes N1 and N2 was 27 mutational steps, suggesting that their genetic relatedness was weak.

3.4. Genetic distance between subpopulations

Based on the results of the phylogeographic analysis, European isolates from 5 countries were treated as a distinct subpopulation of *E. multilocularis*. Among the subpopulations of various localities, the values of pairwise fixation index (Fst) were computed to determine the degree of genetic differentiation (Table 3). The Fst values between Europe and other areas ranged from 0.845 to 0.941, suggesting that it would take many generations after the cessation of gene flow. Similar high values were also obtained between North America and other areas.

3.5. Genetic uniformity of elp gene

The nuclear *elp* gene in *E. multilocularis* represents a single locus [25]. The electropherograms of amplified *elp* gene by direct DNA sequencing showed no double peaks. The concatenated nucleotide sequences of exons VII, VIII and IX in the *elp* gene were completely identical among 74 geographical isolates. The other 2 isolates from Inner Mongolia, which belong to the mitochondrial haplotype O1, showed a single nucleotide polymorphism. In these isolates, a synonymous substitution from A (CAA) to G (CAG) occurred in the position 199 of the exon VIII. The amino acid sequence predicted in this study (197 residues) was the same as that of *E. multilocularis* [12].

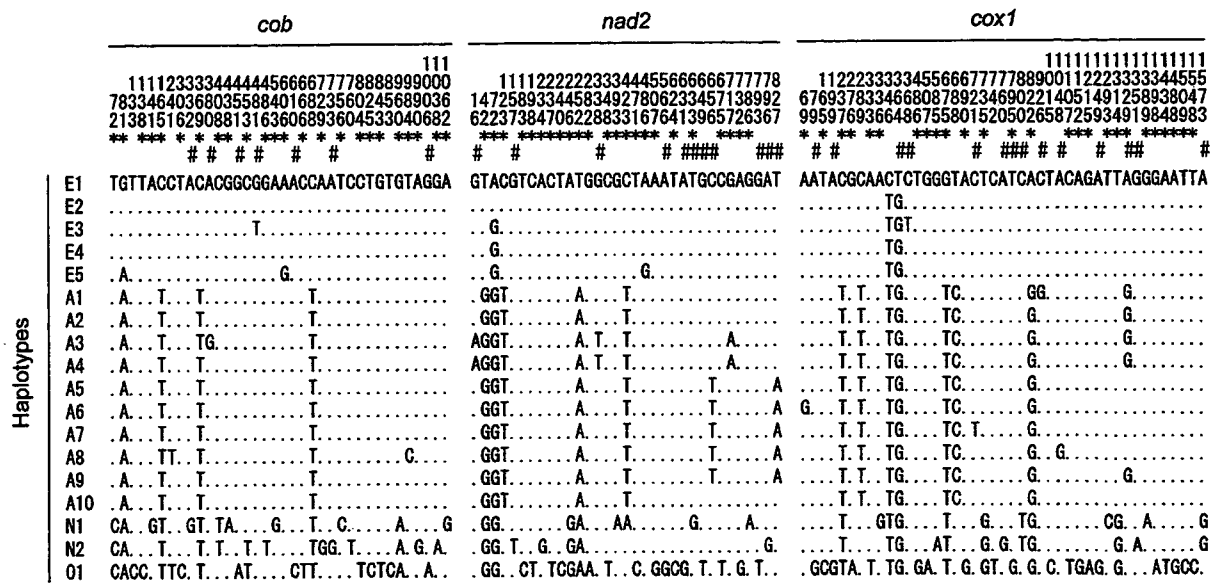

Fig. 1. Nucleotide substitutions of mitochondrial *cob*, *nad2* and *cox1* genes in 18 haplotypes of *E. multilocularis*. The substitutional sites are totally numbered from the initiation codon of each gene. The numbers are shown in vertical. Asterisks indicate the third codon position, and hashmarks denote nonsynonymous substitutions.

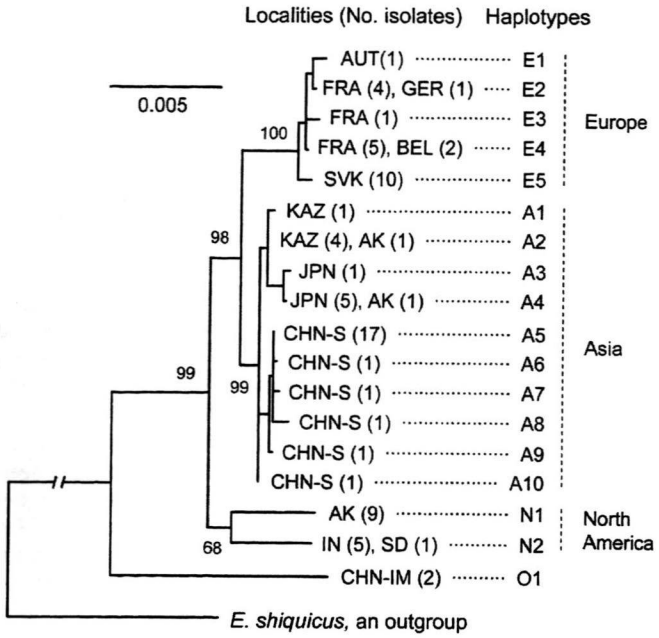

Fig. 2. A neighbor-joining haplotype tree of *E. multilocularis* constructed from the nucleotide data set of mitochondrial *cob*, *nad2* and *cox1* genes. The codes of localities are shown in Table 1. Values on the tree nodes are bootstrap proportions (%). A scale bar (divergence of 0.005) is shown.

By contrast, 2 amino acid replacements were observed when compared with the *elp* protein of *E. granulosus* [26]. The divergence value of the *elp* sequence examined (592 nucleotide sites) reached to 1.40% in pairwise comparison with *E. granulosus*.

4. Discussion

The organisms of the genus *Echinococcus* have a unique reproduction system. The self-fertilization of the hermaphroditic adults and the

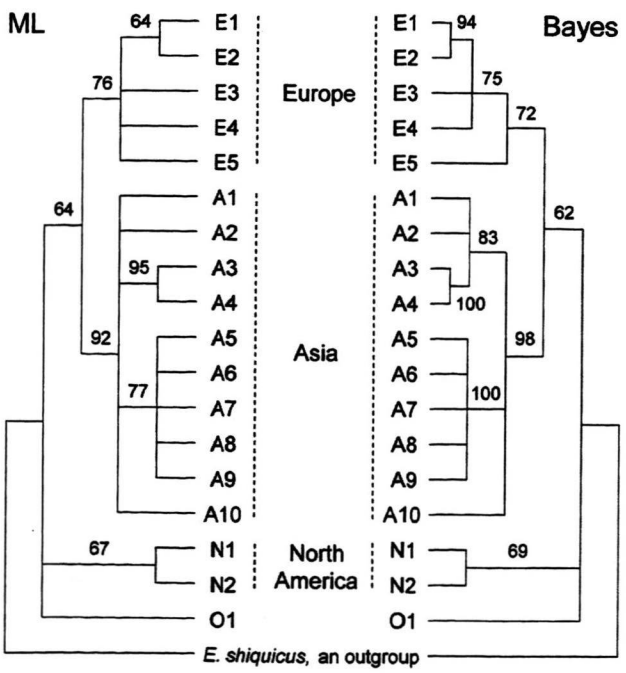

Fig. 3. Haplotype cladograms of *E. multilocularis* inferred by maximum likelihood (ML) and Bayesian methods using the nucleotide data set of mitochondrial *cob*, *nad2* and *cox1* genes. Values in the left tree are ML bootstrap proportions (%), while Bayesian posterior probabilities are shown in the right tree.

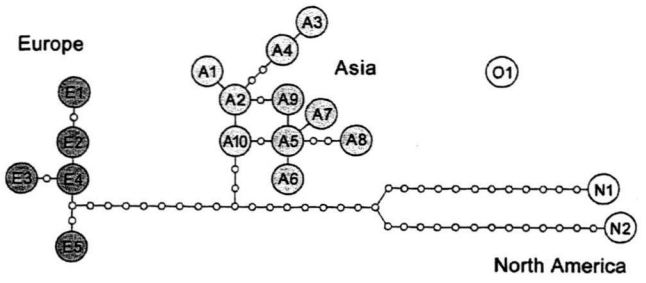

Fig. 4. Mitochondrial haplotype network of *E. multilocularis* based on statistical parsimony. Large circles denote haplotypes found in this study, whereas small circles show hypothetical haplotypes. The haplotype O1 from Inner Mongolia was disconnected with the network.

clonal amplification of the larvae cause the genetic uniformity of local populations [27,28]. Previous studies [6,7] emphasized that the genetic variability of *E. multilocularis* is extremely limited in comparison with *E. granulosus* sensu lato, which showed a large genetic variation [29]. However, recent taxonomic revisions revealed that *E. granulosus* sensu lato is a cryptic species complex including *E. granulosus* sensu stricto, *E. equinus*, *E. ortleppi* and *E. canadensis* [19,30]. The inadequate comparisons, therefore, caused underestimations for the genetic variation of *E. multilocularis*. The other reason for the underestimations was the short lengths of nucleotide sequences examined. The present study succeeded in demonstrating the geographic clades of *E. multilocularis* through the long sequencing of mitochondrial genes.

In this study cladistic approaches clearly divided the local populations of *E. multilocularis* into European, Asian and North American clades, reflecting the possibility of isolation during glacial periods. Our data indicate that the European clade has a sister relationship with the Asian clade, and both clades kept genetic variations at limited levels. The members of the European clade had a restricted distribution, whereas those of the Asian clade prevailed widely. The genetic divergence values between both clades ranged from 0.37 to 0.60%. Because of the lack of a molecular clock in tapeworms, the date of bifurcation into the European and Asian clades was firstly estimated to be 0.30–0.19 million years ago (MYA) by using the standard rate of 2% divergence per million years (Myr) [31]. However, the date corresponding to the Middle Pleistocene epoch (0.78–0.13 MYA) could be overestimated. Recent studies in molecular ecology showed that the rapid substitution rates of mtDNA should be applied for the purpose of analyzing intraspecific relationships [32]. For instance, the faster rates of 6–10% divergence per Myr were used for the phylogeographic analysis of the root vole *Microtus oeconomus* [33], which serves as an intermediate host for *E. multilocularis*. A rapid molecular clock of 10% divergence per Myr estimated that bifurcation into the European and Asian *E. multilocularis* occurred 60,000–37,000 years ago. Even in the use of the rapid clock, the bifurcation predated the Last Glacial Maximum, which took place around 20,000 years ago. The distribution of foxes in the Middle and Late Pleistocene is the most important factor in considering the evolutionary history of *E. multilocularis*, because foxes play a key role to spread the parasite widely. In Europe, the first appearance of red foxes has been retraced into the interglacial epoch Holstein (0.23–0.18 MYA), and in the last glacial period arctic foxes became dominant in Europe [34]. Based on the paleoecological and genetic data, one could speculate that *E. multilocularis* had been introduced into Europe by foxes in the Late Pleistocene (130,000–10,000 years ago). The discontinuous distribution of *E. multilocularis* in recent Europe [1] supports a hypothesis that the European clade is derived from isolated populations in glacial refugia such as the Iberian, Italian and Balkan peninsulas [35].

The most striking feature of our phylogeography is that the Alaskan isolates from the St. Lawrence Island in the Bering Sea were divided

Table 3
Pairwise fixation index (Fst) among *E. multilocularis* subpopulations.

	1	2	3	4	5
1. Europe ($n=24$) [a]					
2. Sichuan, China ($n=22$)	0.905*[c]				
3. St. Lawrence, Alaska ($n=11$)	0.845*	0.843*			
4. Kazakhstan ($n=5$)	0.873*	0.800*	0.714*		
5. Hokkaido, Japan ($n=6$)	0.897*	0.897*	0.757*	0.892*	
6. Central North America ($n=6$) [b]	0.941*	0.979*	0.787*	0.993*	0.994*

[a] Austria, France, Germany, Belgium and Slovakia.
[b] Indiana and South Dakota.
[c] Significant values are indicated by an asterisk ($p<0.05$).

into the Asian and North American clades. In several periods of the Pleistocene, the fall of sea levels by glaciation formed the Bering land bridge, which connected Asia and North America. The bridging land mass called "Beringia" served as a vast refugium covering the East Siberia and Alaska in the Pleistocene ice ages [36]. The unique geological history suggests a possibility that the populations of *E. multilocularis* derived from the Beringia remain as a relic in the St. Lawrence Island. In this study, the same mtDNA haplotypes that appeared in the St. Lawrence Island were also detected in the temperate zones of Kazakhstan and Japan, suggesting that the long-distance dispersal of *E. multilocularis* in the Asian continent occurred during the Holocene to the present. The red fox, which has the ability to adapt easily to various environments [37], might play an essential role for the dispersal. There is a possibility that *E. multilocularis* was introduced into Eastern Hokkaido, Japan by migrant foxes from the Kurile Islands [38].

In central North America we detected the indigenous haplotype N2 from Indiana and South Dakota, which is genetically related with the haplotype N1 from St. Lawrence Island, Alaska. Both haplotypes are important in considering the spread and origin of *E. multilocularis* in North America. A rapid molecular clock of 10% divergence per Myr estimated that the split into the two haplotypes occurred 75,000 years ago. The split largely predated the Last Glacial Maximum, when all of Canada was essentially covered with ice sheet [36]. The recent phylogeographic analysis of the North American red fox demonstrated that populations derived from the refugium of central North America are genetically quite different from the Eurasian and Beringian populations [39]. It seems likely that the vicariance of North American red fox in Pleistocene refugia is directly responsible for the genetic polymorphism of North American *E. multilocularis*. At the present time, *E. multilocularis* is widely distributed in central North America [40,41]. Our data demonstrated that the mtDNA sequences of the isolates from South Dakota and Indiana are completely identical with each other. Moreover, our previous report revealed that a human-derived isolate in Minnesota is closely related with them [42]. Although the number of the isolates examined is insufficient, the genetic homogeneity suggests a possibility that a particular parasite population derived from the refugium of central North America has been widespread by foxes. However, the occurrence of human alveolar echinococcosis in central North America [43,44] is extremely low in comparison with other endemic areas. One could speculate that the infectivity of *E. multilocularis* to humans might be influenced by the intraspecific variation. The experimental infection of rodents with *E. multilocularis* showed that the larval development of Montana isolate was different from that of Alaska isolate [45].

Alveolar *Echinococcus* sp. has been isolated from the corsac fox *Vulpes corsac* in Inner Mongolia [46]. In this study we could analyze two isolates in Inner Mongolia through the courtesy of Chong-Ti Tang, School of Life Sciences, Xiamen University, China. These isolates were classified as the haplotype O1, which retained the most variant mtDNA. However, the values of pairwise divergence between O1 and the other haplotypes were low levels ranging from 1.5 to 2.0% in *cob* and from 1.5 to 1.7% in *cox1*. In the case of the taeniid tapeworm *Taenia solium*, the maximum values of pairwise divergence between members of the worldwide isolates were 2.1% in *cob* and 1.3% in *cox1* [47]. We, therefore, regarded the variation of the haplotype O1 as an intraspecific level. The unique variant in Inner Mongolia is especially valuable in studying the evolutionary history of *E. multilocularis*.

In this study, the nuclear DNA sequence for the exons VII, VIII and IX of the *elp* gene was available to confirm the identification of species because the gene is an informative marker for phylogeny [13]. These exons encode the immunodominant B cell epitope region, which is used as an antigen for the serodiagnosis of alveolar echinococcosis [48]. The nucleotide sequences of the *elp* exons were extremely conservative in the worldwide isolates of *E. multilocularis*, indicating that the *elp* antigen is available for serodiagnosis in any endemic areas.

In conclusion, our data suggest an evolutionary scenario in which distinct parasite populations derived from glacial refugia have been maintained by indigenous host mammals. We postulate that the Beringia and its neighborhood are key regions for the evolution of *E. multilocularis*. More isolates from tundra, taiga and steppe zones are needed to clarify the ancestral origin of *E. multilocularis*. In particular, the phylogeographic analysis for the relatives of the haplotypes O1, N1 and N2 should be conducted in the context of host–parasite interrelationship, including corsac foxes in Central and Northeast Asia, red foxes in North America and arctic foxes in arctic tundra. The incomplete genealogy of *E. multilocularis* may be solved by the resultant genetic data.

Acknowledgements

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Serological Monitoring of Progression of Alveolar Echinococcosis with Multiorgan Involvement by Use of Recombinant Em18$^{\nabla}$

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Two cases of alveolar echinococcosis (AE) with multiple-organ involvement (the liver, lungs, and bone) were monitored by imaging and serology for 20 years. Resection of the bone lesion was complete in one case but incomplete in the other case. Albendazole treatment was markedly to moderately effective against hepatic and pulmonary AE lesions in both cases, whereas it had almost no effect against the bone lesion in one case. The results of the serological tests with recombinant Em18 antigen coincided with the clinical findings in each case. An enzyme-linked immunosorbent assay for the detection of immunoglobulin G (IgG) responses, especially IgG4 responses, is expected to be a real-time indicator of the dynamics of active AE.

Alveolar echinococcosis (AE), caused by the fox tapeworm, *Echinococcus multilocularis*, is one of the neglected, emerging, or reemerging infectious diseases listed by WHO with cysticercosis, rabies, brucellosis, etc., and is often misdiagnosed as hepatocellular carcinoma (4, 17). It is one of the most lethal parasitic infections, and areas contaminated with this parasite are becoming wider and wider in the majority of the Northern Hemisphere, other than tropical and subtropical areas (10, 11, 19, 20, 24). When local people living in contaminated areas accidentally ingest eggs of this parasite expelled from foxes and dogs, the embryos develop into metacestodes, so-called alveolar echinococcus, by asexual proliferation, mainly in the liver (in more than 97% of cases of AE). Infected persons become symptomatic, usually 10 to 20 years later. Patients with active lesions are estimated to die within 15 years after the initial appearance of symptoms, whereas cases with only abortive or inactive AE lesions are rarely found (10, 12, 19, 25). Calcification occurs either at the early stage and requires no treatment (abortive or inactive cases) or at the late stage (the majority of advanced AE cases). Early diagnosis with early treatment, mainly by surgery, has strongly been recommended, since complete excision is so far the only curative treatment (19).

Therefore, the development of sensitive and specific diagnostic tools is crucial. Imaging tools that use ultrasonography, computed tomography (CT), and magnetic resonance imaging have been applied for the diagnosis and monitoring of the progression of AE (19). Most recently, positron emission tomography has also been introduced for the detection and characterization of the active lesions (3).

Serology for the detection of specific antibody responses was developed independently by several groups in Germany, Switzerland, Australia, and Japan by characterization with antigens EM10, Em2/III, EM4, and Em18, respectively (24). It later became evident that all of these were the same ezrin-like protein encoded by *elp* gene, which has a high degree of homology with human ezrin-radexin-moesin (ERM) (2, 10, 22). It is very interesting that specific diagnostic antigen candidates have a very high degree of homology with the host protein (ERM), and AE is a typical chronic disease. Among these four antigens, Em18 is the smallest component of the ezrin-like protein and is degenerated by a cysteine protease(s), but it has the lowest degree of homology with ERM (10, 22). Therefore, serology by the use of Em18, especially recombinant Em18 (recEm18), is expected to show the lowest level of cross-reactivity with human components (1, 5, 7, 8, 10, 13, 17, 22, 24, 25, 28). Furthermore, it has been shown that a positive result by serology with Em18 is a good indicator of active AE, and immunoglobulin G4 (IgG4) is the predominant subclass involved (9, 12).

In this report, we describe two AE cases with lesions in the liver, lungs, and bone who were admitted for surgery of the bone lesion but who showed completely different clinical and serological outcomes after 20 years of monitoring, until the end of 2008. Data on the IgG responses of case patients 1 and 2 detected before 2000 were briefly reported by Fujimoto et al. (5), who designated these patients cases 6 and 7, respectively. In this study, we applied a recEm18-specific enzyme-linked immunosorbent assay (recEm18-ELISA) to the analysis of the serological dynamics of the responses to IgG4, IgG1, IgG (Fab), IgG (H+L), and IgG recognized by recombinant protein G to evaluate which one is the best for monitoring of the progression of AE.

MATERIALS AND METHODS

Serology for the detection of antibodies specific to Em18, the best diagnostic antigen for the detection of active AE, was applied for the monitoring of these

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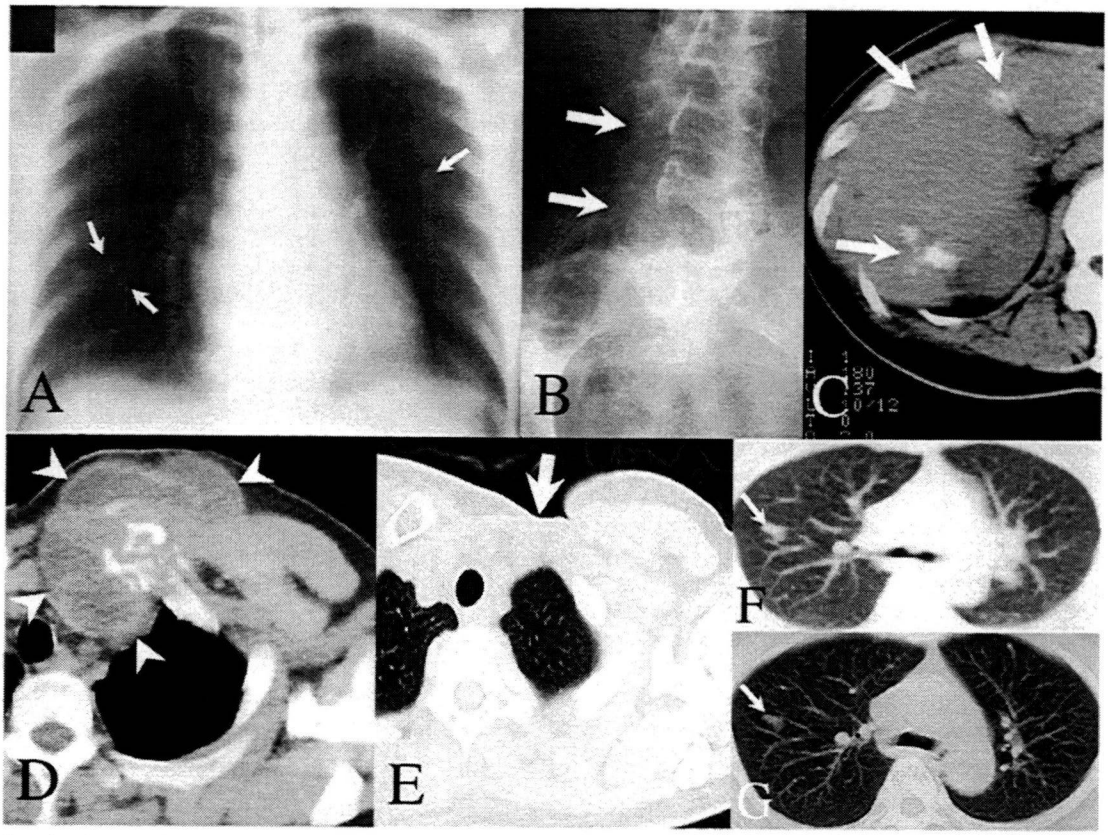

FIG. 1. Imaging figures of case 1 (A to C) and case 2 (D to G). (A) Several nodular densities demonstrated by a chest X-ray tomogram (arrows). (B) Osteolytic destruction of the right lower lumbar vertebrae resulting from AE invasion of the pelvis (arrows), demonstrated by X ray 143 months after ABZ chemotherapy (September 2007). (C) Reduction in size of hepatic AE foci with calcification (arrows), demonstrated 130 months after ABZ chemotherapy (July 2000). (D) Chest CT scan demonstrating a mass with a heterogeneous density (arrowheads) at the left medial head of the clavicle. (E) CT scan image obtained 81 months after radical resection of the bone AE and ABZ chemotherapy (December 1999). A defect after complete removal of the lesion without relapse is demonstrated (arrow). (F) Chest CT scan image demonstrating multiple nodular densities of pulmonary AE (the arrow points to one of the lesions). (G) Chest CT scan image demonstrating multiple nodular densities with few changes 81 months after the time of diagnosis (December 1999) (the arrow points to one of the lesions).

cases (12, 25). The recEm18-ELISA was applied to the analysis of the dynamics of the antibody titers throughout the treatment (22, 25). The serum samples used for the ELISA for either total IgG, IgG1, or IgG4 were used at a 1/200 dilution, whereas those used for immunoblotting (IB) were used at a 1/50 dilution (9, 12). Final ELISAs by the use three different tools were carried out in 2008 under the same conditions to show the dynamic changes throughout the follow-up studies. Five secondary antibodies were used for these ELISAs: horseradish peroxidase-recombinant protein G (Zymed Laboratories Inc.), horseradish peroxidase-monoclonal anti-human IgG1 and IgG4 (Zymed Laboratories Inc.), and peroxidase-conjugated goat IgG fraction to human IgG (Fab) and IgG (H+L) (Cappel).

RESULTS

Clinical follow-up. (i) Case 1. A 39-year-old woman was admitted to Asahikawa Medical College Hospital (AMCH) in August 1989. She lived in the eastern part of Hokkaido, known as the area of Japan where AE is the most endemic. On admission, she had a painful tumor in the right buttock with pus oozing through a fistula. Initially, beginning in September 1987, she felt only a dull pain in the right buttock, but she had a lump about the size of her fist afterwards. Even though the softened part of the lesion had been incised and drained during a previous hospitalization in April 1988, intractable pyorrhea followed. She underwent curettage of the sequestrum for chronic suppurative osteomyelitis of the ileum in July of the same year. She was then given antitubercular drugs owing to a misdiagnosis of tuberculous osteomyelitis, without effect. Another curettage of the sequestrum was performed in November 1988, but she was not cured of the persistent pyorrhea. For that reason, she was transferred to AMCH for a close examination of the refractory bone infection. Histological findings specific to AE were disclosed by microscopic examination of a biopsy specimen obtained at the time of the previous surgery. Multiple AE lesions were consequently detected in the liver (data not shown) and the lungs (Fig. 1A) by imaging diagnosis. Laparoscopy and a direct-vision hepatic biopsy revealed the endoscopic and histopathological picture of AE. She was finally diagnosed with AE involving the right ileum, the liver, and the lungs. As radical surgery of all the involved organs was considered impossible, administration of albendazole (ABZ; 400 mg orally twice daily) started on 18 October 1989 (6, 18, 23, 27). Continuous medication was opted for because of the severe condition of her illness, even though the intermittent administration of ABZ (a 28-day cycle followed by a 14-day ABZ-free interval) is the recommended regimen. The prolonged drainage stopped 6 weeks after the start of chemother-

apy. She underwent radical curettage of the sequestrum and bone grafting for the right ileum AE on 19 December 1989. As her clinical course was good for 3 years after the operation, she was not treated with ABZ from March 1993 to March 1994. However, the ileum AE recurred after the stop of chemotherapy, and ABZ chemotherapy was resumed, followed by a second operation. She underwent a third operation for AE invasion of the fifth lumbar vertebra in October 1999, and the dose of ABZ was increased to 600 mg. The dose of ABZ was returned to 400 mg due to mild liver dysfunction in September 2000, and intermittent medication was chosen 1 year later. Although pain in the right leg developed with the gradual progression of the bone AE, it subsided for several months with the use of continuous medication. She has so far needed surgical treatment four times and gradual increases in the dose of ABZ. Despite the combined medical and surgical treatment, the bone AE has hardly been controlled (Fig. 1B). Furthermore, she complained of bloody sputum in January 2005, and diagnostic imaging revealed the recurrence of pulmonary AE. However, this respiratory symptom was controlled by use of an increased dose of ABZ (1,000 mg daily), which had no marked adverse effects. In contrast, the hepatic AE remained in complete remission (Fig. 1C) and has not recurred.

(ii) Case 2. A 41-year-old woman living in the central part of Hokkaido felt a pain in her upper left chest at the end of July 1991. She was first diagnosed with sternoclavicular joint arthritis and shoulder periarthritis at AMCH on 14 August 1991. A tumor of 50 by 60 mm in the sternoclavicular joint was found by physical examination on 19 September 1991 (Fig. 1D). The pain disappeared when the tumor became large, and this was accompanied by central necrosis. A cytological examination and bacterial culture of an aspirate were performed on 13 February 1992. However, there were neither malignant cells nor bacterial growth. An open biopsy was performed on 12 February 1993, and a histopathological diagnosis of AE was made by microscopic examination. Multiple AE lesions in both the liver and the lungs (Fig. 1F) were subsequently detected by imaging diagnosis. She was diagnosed with AE with multiorgan involvement (the liver, the lung, and the clavicle) after 20 months of upper chest pain. She underwent radical resection of the left clavicular AE lesion (Fig. 1E). In addition, AE involvement of the liver was confirmed by laparoscopy and ultrasound-guided liver biopsy (data not shown). Chemotherapy with ABZ against AE of the liver and lungs started on 1 April 1993. After a remarkable reduction in the size of the hepatic AE lesion was detected by CT after 1 year with this chemotherapy, we recommended that the ABZ treatment be stopped and that the AE lesions be monitored. The hepatic AE remained in complete remission. However, the lung lesions may still have been viable and growing, as determined by CT on 4 January 1996 (data not shown). As summarized in serological follow-up studies (Fig. 2B), serology still indicated weakly positive results. We therefore resumed ABZ treatment from March 1996, and no exacerbation of AE lesions either in the lungs (Fig. 1G) or in the liver (data not shown) was detected by diagnostic imaging after that.

Serological follow-up. The clinical background information and serological data obtained by recEm18-ELISA are shown in Fig. 2. The antibody response to recEm18 showed highly dynamic and variable changes over 20 years, until October 2008, for case 1, who was referred to as case 7 before 2000 in the work of Fujimoto and others (5) (Fig. 2A). There was a certain correlation between the ELISA results and her clinical course mentioned above. The ELISA values remained positive after palliative surgeries of the iliac lesion and rose during the advanced stages of the disease, especially after 2000. We noticed a rise in the ELISA values after the third operation, and in October 2000, she complained of pain in the right hip and a relapse of the right iliac AE was found. It was supposed that the rise in the ELISA values mainly originated at the focus of the infection, because the increased ELISA values remained after the imperfect operation and because a relapse or the development of new AE lesions in other organs was not observed. In this study, we carried out ELISA for the detection of IgG4 and IgG1 (data not shown) as well as total IgG using several secondary antibodies, including recombinant protein G, anti-IgG (Fab), and anti-IgG (H+L). As shown in Fig. 2, it was clear that the ELISA values for the detection of antibodies specific to recEm18 by the use of recombinant protein G, anti-human IgG (Fab), and anti-human IgG4 were highly reliable for monitoring of the clinical courses. There were no critical differences in the antibody responses when anti-human IgG (Fab) and anti-human IgG4 were used. As the IgG1 responses were rather weak in these two cases and the IgG response that was detectable with anti-human IgG (H+L) showed highly nonspecific background responses, these results were not included in Fig. 2 and are not useful for the monitoring of progression of the disease (data not shown). Among these five tools, the detection of IgG4 was expected to be the most sensitive for follow-up studies.

When we applied the recEm18-ELISA to case 2 (Fig. 2B), a drastic drop in antibody titers was confirmed within 4 years before 2000 (5). In this study, we monitored the patients until October 2008. The IgG response still appeared to be positive until 2000. However, when we checked the IgG4 response, it was negative at that time and remained negative until October 2008. The result of the IgG-ELISA became negative by 2003 and remained negative until October 2008. All clinical findings, including those obtained by ultrasonography and magnetic resonance imaging, also supported a diagnosis of a complete cure, which corresponded to the serological data in this case.

DISCUSSION

The inoperable parasitic lesion of the bone in case 1 progressed even after continuous chemotherapy for 20 years, starting in 1989. This is because of the inoperable residual lesion in the bone. In contrast, case 2 is expected to have been cured after complete surgical removal of the lesion in the bone. Chemotherapy with ABZ appeared to be markedly to moderately effective against hepatic and pulmonary AE in these two cases. In contrast, more prolonged treatment may be required for AE at sites such as bone and the brain, as clearly described in the summary of product characteristics by SmithKline Beecham. The proportion of surgically resectable cases of AE was 40% or less, and the mortality rate within 10 years for patients with nonresectable cases was 90% in about 1980 (23). Therefore, the prognoses for these two AE patients with multiorgan involvement were presumed to be very poor, but they have survived over a long period of time. These outcomes are

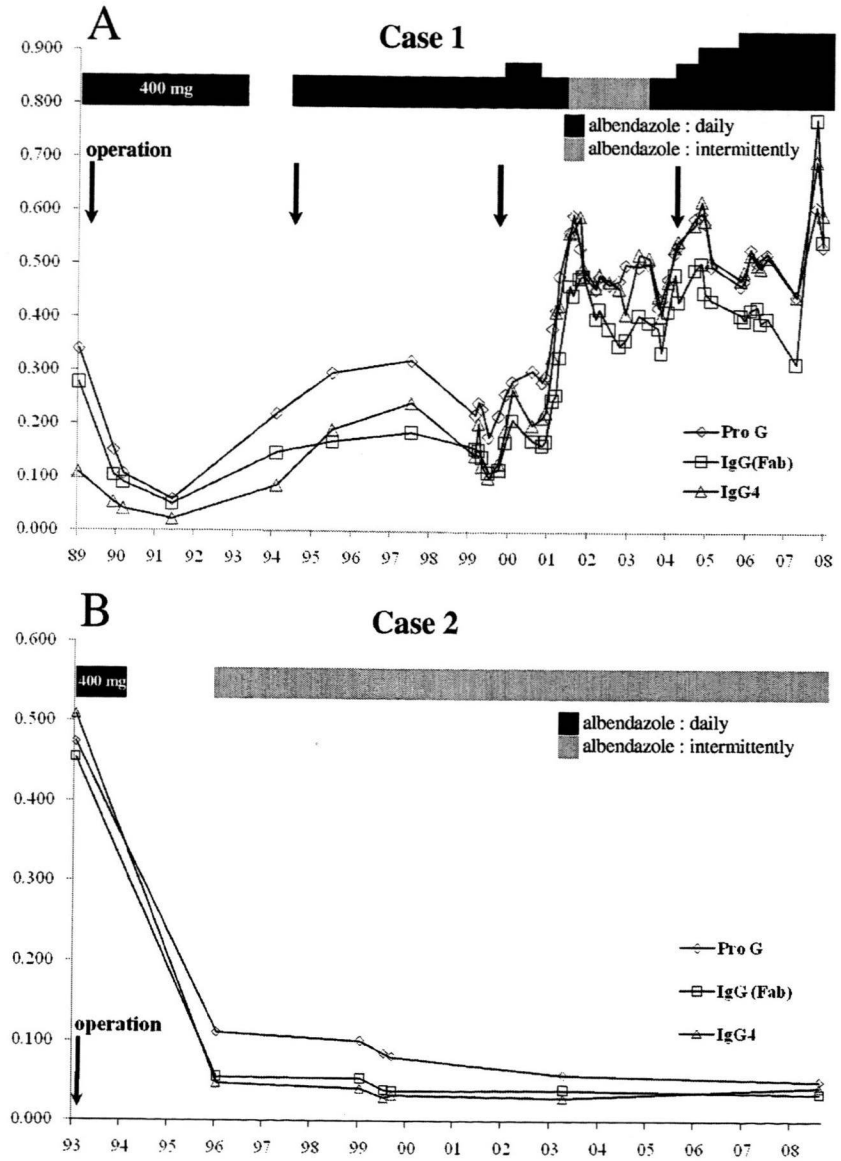

FIG. 2. Antibody responses to recombinant Em18 over the course of follow-up of the two AE cases. Four different secondary antibodies (anti-human IgG [Fab], anti-human IgG [H+L], anti-human IgG1, and anti-human IgG4) and recombinant protein G (Pro G) were applied for these follow-up studies. The data for the IgG (H+L) and IgG1 responses are not included.

very encouraging for patients needing long-term ABZ chemotherapy for the treatment of severe AE.

WHO has recommended combined imaging and serology for the diagnosis of AE and cystic echinococcosis (19). Imaging diagnosis of AE is comparatively easy in cases with distinctive findings, such as calcification and cavitations of lesions in patients with advanced AE, especially in areas of endemicity, although it is not always possible to make a conclusive specific diagnosis from imaging figures only. The addition of highly specific serology is strongly recommended. The use of Em2^{plus}-ELISA, recEm18-IB, and/or recEm18-ELISA, as well as several other ELISAs, has been recommended by WHO (19). However, a recent comparative analysis of the recEm10-ELISA, Em2^{plus}-ELISA, and recEm18-ELISA for monitoring of the progression of different pathological types of AE by the WHO criteria (14, 16, 25) revealed that the recEm18-ELISA exclusively showed strongly dynamic changes, including negative results for cured cases (25). The sensitivity of either recEm18-ELISA or RecEm1-IB was superior to that of other serological tools, including the Em2^{plus}-ELISA (1, 10, 25). Similar data were observed before and after liver transplantation for AE cases in France (S. Bresson-Hadni and A. Ito, unpublished data) and follow-up studies of hepatic AE cases with radical resections in Japan (5; H. Akabane and A. Ito, unpublished data). The most remarkable picture provided by the recEm18-ELISA is that the real-time increases in ELISA values correlated with relapses. As serology by either Em18-IB or Em18-ELISA was highly useful for the detection of approximately 95% of active AE cases (1, 10, 12, 21, 25), it is

further notable that the results becomes negative after a complete cure (5, 12, 13, 25; J. F. Wilson, P. M. Schantz, and A. Ito, unpublished data). After curative surgery, the results may become negative within a half year (Akabane and Ito, unpublished). Therefore, it is strongly recommended that the recEm18-ELISA by applied, especially for the detection of IgG4, for monitoring of the progression of AE. Several reports have stressed the usefulness of the detection of IgG4 (12, 26), as serum samples that gave optical density values at 405 nm that were greater than the mean ± 3 standard deviations were considered to be seropositive, Although IgG4 responses became negative a little bit faster than the IgG (Fab) responses (Fig. 2B), we consider that there is no crucial difference between the detection of IgG4 and IgG (Fab).

In order to detect the antibody response for outpatients or screening of patients in areas where AE is endemic, as well as inpatients, a rapid immunochromatographic test (ICT) which does not need any special facilities or experienced personnel has already been developed by the use of recEm18 (21). The disease in the two AE cases described here was also confirmed by ICT (data not shown). A commercially available ICT kit is ready from Adtec Inc. (Usashi, Oita, Japan). A quantitative ICT is under development for monitoring of the progression of AE (Y. Sako et al., unpublished data).

Although ABZ is still the preferred chemotherapeutic agent for the treatment of AE, continuous dosing may be essential (15), since some resistance to ABZ appeared to develop after the cessation of ABZ treatment (19). As demonstrated in this study, Em18 serology is a good marker for the presence of active AE lesions and inactive or abortive AE cases, as well as for the detection of seronegativity in cured AE cases after radical surgery. However, ABZ does not usually have a clear metacestocidal effect in vivo or clinically (15), and an indication of inactive or abortive AE does not always mean a complete cure (Bresson-Hadni and Ito, unpublished). Without direct evidence that an AE lesion is dead or completely calcified and has no germinal layer through histopathological examination of biopsy specimens or radical resection of whole lesions, it is still difficult to recommend when chemotherapy for AE cases should be stopped. Standards for the time of cessation of ABZ treatment remain unresolved, and the development of metacestocidal drugs is still needed.

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T.M. did surgery at the affiliated hospital of AMCH; and Y.I., T.O., Y.K., Y.O., and T.M. followed the clinical treatment after surgery at the affiliated hospital of AMCH and Ishikawa Clinic. Y.S., S.I., Y.I., and A.I. did the serology. Pathological examination was carried out by N.M., K.N., and M.N., Y.I., T.O., Y.K., and A.I. prepared the manuscript. All authors read through the manuscript.

We received written agreement from the two patients.

We declare that we have no conflicts of interest.

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PLoS NEGLECTED TROPICAL DISEASES

Natural Infection of the Ground Squirrel (*Spermophilus* spp.) with *Echinococcus granulosus* in China

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Abstract

Background: *Echinococcus granulosus* is usually transmitted between canid definitive hosts and ungulate intermediate hosts.

Methodology/Principal Findings: Lesions found in the livers of ground squirrels, *Spermophilus dauricus/alashanicus*, trapped in Ningxia Hui Autonomous Region, an area in China co-endemic for both *E. granulosus* and *E. multilocularis*, were subjected to molecular genotyping for *Echinococcus* spp. DNA. One of the lesions was shown to be caused by *E. granulosus* and subsequently by histology to contain viable protoscoleces.

Conclusions/Significance: This is the first report of a natural infection of the ground squirrel with *E. granulosus*. This does not provide definitive proof of a cycle involving ground squirrels and dogs or foxes, but it is clear that there is active *E. granulosus* transmission occurring in this area, despite a recent past decline in the dog population in southern Ningxia.

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Introduction

The canid adapted intestinal tapeworms, *Echinococcus granulosus* and *E. multilocularis* are important zoonotic pathogens that cause serious disease in humans [1]; both are endemic to Ningxia Hui Autonomous Region (NHAR) in northwest China [2,3]. *E. granulosus* can be transmitted through either sylvatic cycles, involving wild carnivores and ungulates; or via domestic cycles, usually involving dogs and farm livestock. A common source of infection for dogs is hydatid infected offal from sheep, which often harbour the common G1 genotype (sheep-dog strain) responsible globally for most cases of human cystic echinococcosis (CE) [1,4]. *E. multilocularis* is primarily maintained in a sylvatic life-cycle between foxes and rodents, with human infections considered as a relatively rare accidental event caused by spill-over from the wildlife cycle in European countries [5]. Synanthropic transmission cycles are believed to be responsible for the high prevalence of human alveolar echinococcosis (AE) in Alaska and on the eastern Tibetan Plateau, whereby domestic dogs predating on rodents in and around villages are considered to be the primary source of infection causing human AE [6,7].

A report of *E. granulosus* in plateau pika (*Ochotona curzoniae*) in Qinghai Province [8] appears retrospectively almost certainly to be due to *E. shiquicus*, a new *Echinococcus* species described in 2005 that infects Tibetan foxes (*Vulpes ferrilata*) on the Tibetan Plateau [9]. Work in the 1980s in NHAR indicated that the transmission modes for co-hyperendemic AE and CE involved domestic dogs/livestock (mainly sheep) for CE and foxes/rodents for AE [10].

Extensive investigations that we undertook in 2001–2007 to update available epidemiological data and to monitor the transmission patterns of both *E. granulosus* and *E. mulilocularis* in NHAR, indicated that owned dogs were a risk factor for human AE (involving a dog/rodent cycle) as well as CE (involving a dog/domestic livestock cycle) [3,11]. An increase in susceptible rodent populations due to deforestation and over use of farmland for agriculture have been emphasised as important zoonotic risk factors for human AE in NHAR and in other Chinese settings [11,12]. As part of these ongoing studies, we captured small mammals on the southern slopes of Yueliang Mountain, Xiji County (Figure 1) (E, 105°64′–105°89′; N, 36°03′–36°18; altitude ranging from 2000–2200 m) in July, 2007. This is an area known to be co-endemic for both human AE and CE [3], and where high seroprevalence for echinococcosis among village-children has been recorded [13]. Of 500 trapped small mammals (mainly ground squirrels; *Spermophilus dauricus/alashanicus* referred to also as *S. dauricus*, *Myospalax fontanieri* and *Mus musculus*), macroscopic cyst-

Author Summary

Echinococcus granulosus and *E. multilocularis* are important zoonotic pathogens that cause serious disease in humans. *E. granulosus* can be transmitted through sylvatic cycles, involving wild carnivores and ungulates; or via domestic cycles, usually involving dogs and farm livestock. *E. multilocularis* is primarily maintained in a sylvatic life-cycle between foxes and rodents. As part of extensive investigations that we undertook to update available epidemiological data and to monitor the transmission patterns of both *E. granulosus* and *E. mulilocularis* in Ningxia Hui Autonomous Region (NHAR) in northwest China, we captured small mammals on the southern slopes of Yueliang Mountain, Xiji, an area co-endemic for human alveolar echinococcosis and cystic echinococcosis. Of 500 trapped small mammals (mainly ground squirrels; *Spermophilus daüricus/alashanicus*), macroscopic cyst-like lesions (size range 1–10 mm) were found on the liver surface of approximately 10% animals. One of the lesions was shown by DNA analysis to be caused by *E. granulosus* and by histology to contain viable protoscoleces. This is the first report of a natural infection of the ground squirrel with *E. granulosus*. We have no definitive proof of a cycle involving ground squirrels and dogs/foxes but it is evident that there is active *E. granulosus* transmission occurring in this area.

like lesions (size range 1–10 mm) were found on the liver surface of approximately 10% animals. Lesions were subjected to molecular genotyping and histopathological examination. None were attributable to *E. multilocularis* but one lesion was identified unambiguously as *E. granulosus*, subsequently shown by histology to contain viable protoscoleces. This is the first report of a natural infection of the ground squirrel with *E. granulosus*.

Materials and Methods

This study was reviewed and approved by the Ethics Committee of Ningxia Medical University. All small mammals were humanely euthanized soon after being trapped. Animals were identified, dissected and the obtained livers fixed in absolute ethanol for DNA analysis and histology.

Prior to histopathology, involving sectioning, haematoxylin/eosin staining and microscopic examination by standard procedures, liver lesions were transported to the Cestode Zoonoses Laboratory (University of Salford, U.K.) for molecular genotyping. Genomic DNA was extracted from these lesions using the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and used as a template for the amplification of a fragment within the mitochondrial 12S rRNA gene [14,15]. Amplified cestode-specific DNA was gel purified using the PureLink™ quick gel extraction kit (Invitrogen, Paisely, U.K.) and commercially sequenced (Cogenics, Takeley, U.K.). The identity of one of these samples was confirmed in another laboratory (Department of Parasitology, Asahikawa Medical College, Asahikawa) by partial sequencing of the mitochondrial *cox1* gene as described [16].

Results

Comparison of the generated sequence data with those held on the NCBI database (www.ncbi.nlm.nih.gov) through the use of BLAST

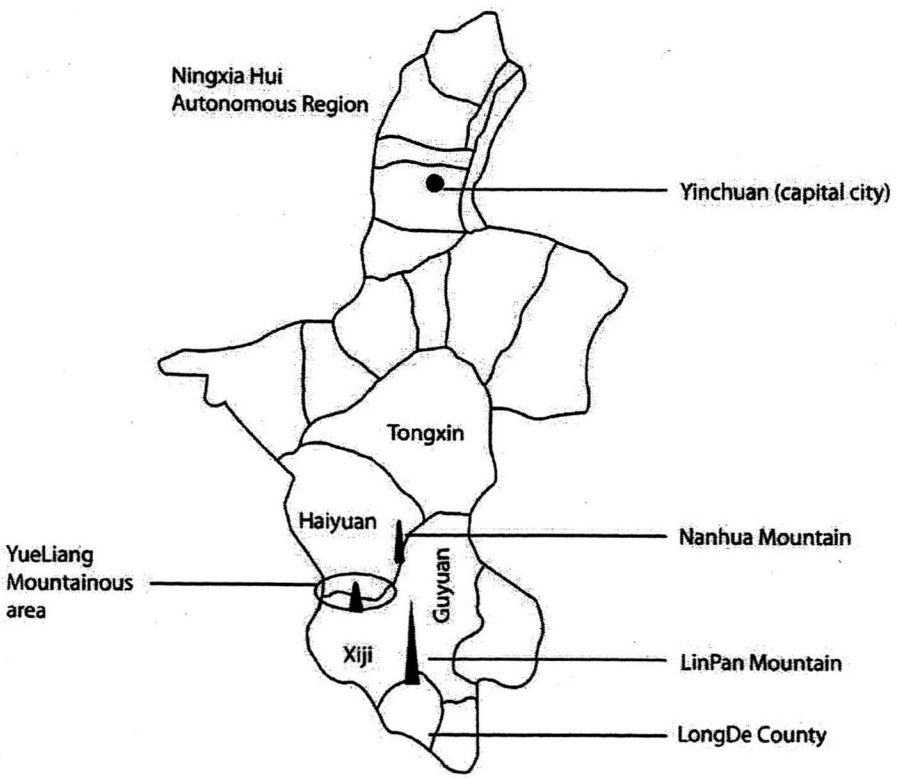

Figure 1. Map of the Yueliang Mountain area in Southern NHAR, China.
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program revealed one sample had 100% homology (254 bp) with the mitochondrial 12S rRNA gene of *E. granulosus* genotype G1 (common sheep-dog strain) (accession nos. DQ408422, AF297617, AB031350, AB024515). Compared with previously published gene sequences (AF297617, *E. granulosus* G1 (common sheep-dog strain); AB 018440, *E. multilocularis*), *cox1* sequence (789 bp) for the sample was nearly identical to that of the published *E. granulosus* G1 sequence with the exception that three transitional changes were present at positions 243 (G/A), 530 (C/T) and 594 (T/C) for the isolate. The sequence shared only 80% identity with the published *E. multilocularis cox1* gene sequence.

Subsequent histological examination of this ground squirrel liver lesion revealed the presence of a thick laminated layer, thin germinal layer and presence of brood capsules containing viable *E. granulosus* protoscoleces (Figure 2).

Discussion

There are numerous previous reports of small mammal species infected with *E. multilocularis* in China and Europe [12,17]. It is well accepted that microtine rodent species are the main reservoir hosts of *E. multilocularis*, though other rodent groups and even lagomorphs (hares and pikas) may also be naturally infected [6,18]. However as far as we know, apart from experimental infection of rodents using either protoscolex or oncosphere injection [19,20], or oral administration of viable eggs [21], this is the first report of a rodent species naturally infected with the metacestode stage of *E. granulosus*. Other non rodent small mammals harbouring lesions of *E. granulosus* (identified morphologically) have been described in hares in Argentina [22] and rabbits in Australia [23,24]. The current observation has shown, for the first time, the rodent, *Spermophilus dauricus*, is susceptible and can be infected naturally with *E. granulosus* that can become viable, producing fertile cysts.

Land cover in the southern mountainous areas of NHAR has undergone important changes since the second half of the 20th century. The area was largely deforested in the 1970s–80s, and in the rolling hills around the southern Liupan Shan, total tree clearance was completed in the mid 90s. Now, the landscape consists entirely of fields for production of wheat, potatoes, beans, alfalfa, etc. During the late 1970s valleys and lower slopes were generally used for agricultural crop production while the upper slopes and hill tops were reserved for grazing. At the time there were no livestock restrictions and grazing pressure was intense. In the late 90s, massive reforestation campaigns carried out to prevent soil erosion led to extensive re-planting of trees and restrictions in sheep numbers allowable per family. The landscape changes had a subsequent major effect on small mammal communities [25] and may have played a role modifying cestode transmission patterns involving small mammals. For instance the opening of the landscape during the deforestation process may have increased the area of habitats favourable to *Spermophilus dauricus* [25]. On the other hand, the dog population has been recovering after the banning of indiscriminate rodenticide use in NHAR from 2002 [12]. The high susceptibility of various host species present together with high parasite prevalence may have increased the infection of definitive and intermediate hosts for both *E. granulosus* and *E. multilocularis*. It is possible that free roaming dogs not only could get infected with *E. granulosus* after feeding on discarded sheep offal containing larval *E. granulosus* but also perhaps through predation on *Spermophilus dauricus*. This large rodent species is one of the commonest in the area and largely occurs in fields, fallows and in the early stages of re-forestation. The red fox (*Vulpes vulpes*) which mostly feeds on small mammals is also a potential candidate for *E. granulosus* transmission in this area of NHAR since this canid species has been shown to be susceptible by experimental infection [11], although it usually harbours smaller worm burdens than dogs, and it has been found naturally infected in Australia and Europe [26–31].

Although we have no definitive proof of a cycle involving ground squirrels and dogs/foxes, it is clear there is active *E. granulosus* transmission occurring in this area, despite the recent past decline in the dog population in southern Ningxia [3,12]. Possible misidentification of morphological specimens of *Echinococcus* obtained from small mammals may have occurred in the past [10]. Therefore, in further epizootiological surveillance of echinococcosis, it would be useful to apply DNA typing of metacestodes from small mammals and copro-DNA techniques [32] for unambiguous identification of fox or dog infections in order to provide accurate baseline data on transmission and to inform a model [33,34] for future integrated control options.

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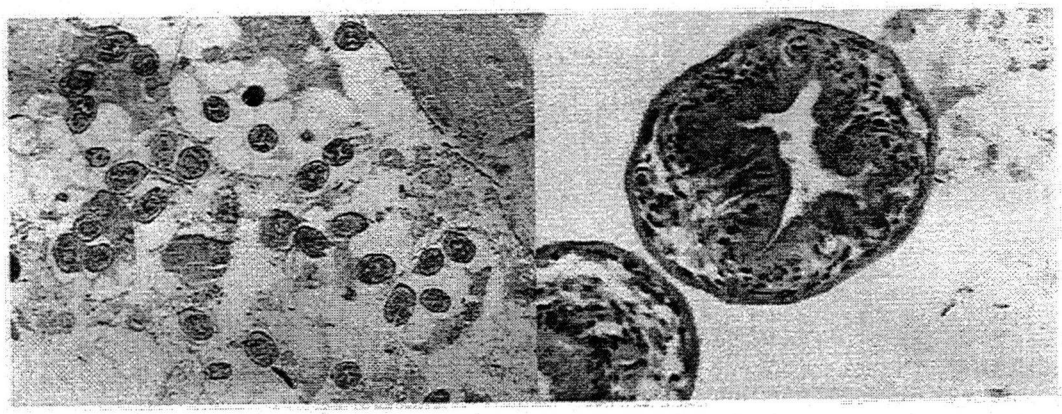

Figure 2. Histopathologic section (haematoxylin and eosin stain) of the liver lesion from a ground squirrel (*Spermophilus dauricus/alashanicus*). Left panel shows the typical appearance of a fertile *Echinococcus granulosus* cyst with laminated and germinal layers, brood capsules and numerous viable protoscoleces; right panel is an enlarged part of the section showing two viable protoscoleces (×1000).
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Author Contributions

Conceived and designed the experiments: YRY TL PSC AI PG DPM. Performed the experiments: YRY TL XB BB MN AI JZZ. Analyzed the data: YRY TL XB BB PSC MN AI JZZ PG DPM. Contributed reagents/materials/analysis tools: MN JZZ. Wrote the paper: YRY BB PSC AI PG DPM.

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ORIGINAL PAPER

Cystic echinococcosis in Turkey: genetic variability and first record of the pig strain (G7) in the country

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Abstract A sample of 22 *Echinococcus granulosus* isolates collected from 12 sheep and ten humans from a focus of cystic echinococcosis in western Turkey was examined by DNA sequencing of four mitochondrial genes (*cox1*, *atp6*, *nad1*, *rrnS*). Results demonstrated the presence of two species of *E. granulosus* complex, *E. granulosus* sensu stricto and *E. canadensis*. Of *E. granulosus* sensu stricto, the G1 genotype (including three microvariants) was found in 17 isolates from humans and sheep, the G3 genotype and an intermediate form G1/G3 in one isolate each (both from sheep). Of *E. canadensis*, the pig strain G7 was found in three isolates from sheep and human. This is the first report of this strain in Turkey. Its presence has implications for local control programs due to its shorter maturation rate in dogs compared with *E. granulosus* sensu stricto. Goat and/or wild boar are likely reservoirs for G7 in the region. We provided further data on the pattern and frequency of nucleotide substitutions within the G1/G3 cluster. Based on our results and GenBank records, G2 (Tasmanian sheep strain) is not considered as a discrete genotypic unit, as its sequences at polymorphic sites conform to microvariants of both G1 and (more often) G3.

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Introduction

Cystic echinococcosis (CE) is a zoonotic, potentially life-threatening infection. This near-cosmopolitan disease constitutes a public health problem in many regions. The life cycle of the causative agents of CE, members of the *Echinococcus granulosus* complex are transmitted between carnivorous definitive hosts such as dogs and wild canids and herbivorous intermediate hosts in which the metacestode (hydatid cyst) develops.

At least ten genotypically defined strains (G1–G10) were described within the *E. granulosus* complex, some of which exhibit marked biological and morphological differences. Such genotypes were recently proposed to merit species status, namely *E. granulosus* sensu stricto (G1–G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6–G10) (Thompson and McManus 2002; Lavikainen et al. 2003; Thompson et al. 2006; Nakao et al. 2007; Moks et al. 2008). In addition, *E. felidis* perpetuating in Africa was recently suggested to be an independent taxon (Hüttner et al. 2008).