Short Report: Histopathological, Serological, and Molecular Confirmation of Indigenous Alveolar Echinococcosis Cases in Mongolia

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Abstract. Alveolar echinococcosis cases diagnosed histopathologically in 2002, 2006, 2007, and 2009 in Ulaanbaatar, Mongolia were reconfirmed by evaluating the cytochrome *c* oxidase subunit I gene of mitochondrial DNA. The most recent three cases using paraffin-embedded and ethanol-fixed specimens revealed that one was of the “Asian” haplotype, whereas two others were of the “Inner Mongolian” type. All patients were born in the western provinces of Mongolia, they never resided outside of Mongolia, and they were given a preliminary diagnosis of malignant hepatic tumor or abscess. The most recent two cases were also confirmed serologically to be active alveolar echinococcosis.

Alveolar echinococcosis (AE), often misdiagnosed as hepatocellular carcinoma, is caused by the accidental ingestion of eggs of the fox tapeworm, *Echinococcus multilocularis*. AE is one of the most lethal parasitic zoonoses and is prevalent in most areas of the Northern Hemisphere. Endemic areas include parts of North America (Canada, Alaska, and some of the lower contiguous states of the United States),¹ Asia (Russia and other states of the former Soviet Union),^{1,2} China,^{1,3–5} and Japan^{1,6}), and the majority of Europe.^{1,7} One of the largest known foci of AE is in China.^{1,3–5} Although *E. multilocularis* has been reported from Russia^{1,2} and China,^{1,3–5} only two AE cases have previously been reported from Mongolia (the first case in 1982 and the second in 2002).^{8,9}

Recently, we have confirmed three additional indigenous AE cases at the State Central Clinical Hospital (SCCH) in Ulaanbaatar (UB), Mongolia in 2006, 2007, and 2009 with ethical approvals in Mongolia. Here, we summarize the three most recent AE cases as well as the case from 2002. Cases were confirmed by histopathological observation (Figure 1), serology (Figure 2), and molecular analysis based on cytochrome *c* oxidase subunit I (*cox1*) of mitochondrial DNA (Figure 3).

Case 2, a 28-year-old policeman born in Orkhon-Uul province, was admitted to the SCCH in 2002. Pre-surgical diagnosis was a suspected chronic liver abscess. During surgery, the patient died of heart failure. Post-mortem diagnosis was hepatic AE with a 13.8 × 7.7-cm lesion on the right lobe of the liver.

Case 3, a 25-year-old disabled man born in Uvs province but living in UB since 1995, was admitted to the SCCH in 2006. He had a 15 × 9.5-cm lesion on the right lobe of the liver with invasion to the diaphragm. The lesion was not resectable, and he died 5 days post-operatively from liver failure. Post-mortem diagnosis was hepatic AE.

Case 4, a 22-year-old female student born in Khovd province, was admitted to the SCCH in May 2007. She had a 7 × 7.5-cm lesion on the left lobe of the liver and a 4 × 4.5-cm lesion on the upper lobe of the left lung. The hepatic lesion was resected but was deemed too large for radical resection, and therefore, the pulmonary cyst was left without surgical treatment. Histopathology revealed hepatic AE (Figure 1).

Case 5, a 20-year-old unemployed female born in Bayan-Ulgii province, was admitted to the SCCH in January 2009 with hepatic and pulmonary lesions. She had a 6.3 × 6.2-cm lesion on the right lobe of the liver. Histopathology revealed hepatic AE. Among the five confirmed AE cases in Mongolia, three patients (cases 1–3) died before, during, or within 1 week of surgery because of the advanced stage of disease.⁸

A serum sample from case 4 was obtained in September 2008, 1 year after surgery, without any clinical background information. Serology (Figure 2) was carried out by immunoblot (IB; Figure 2A) and a commercially available rapid immunochromatography (ICT) kit¹⁰ (Figures 2B–C) using a recombinant Em18 (RecEm18).^{11,12} Case 4 showed very strong antibody responses to RecEm18, the highly specific diagnostic antigen for detection of active AE by both IB and ICT.^{10–15} This finding indicates that this woman still had active lesions, because serology becomes negative within 1 year of successful radical resection.^{12,13} A pre-surgical serum sample from case 5 also showed very strong antibody responses to RecEm18 in IB and ICT, suggesting that AE could have been easily confirmed if serology was introduced for diagnosis before surgery. Antibody responses in case 4 seemed to be much stronger than that in case 5 when we tested these sera by the rapid ICT kit (Figure 2B–C), because the strength of the band is a quantitative result (Sako Y and others, unpublished data). All known Mongolian AE cases,^{8,9,16} including the three most recent cases, were diagnosed as malignant hepatic tumors or abscesses before surgery, but they were confirmed as AE after histopathological examination.

A formalin-fixed specimen was evaluated for the case diagnosed in 2002 (case 2), paraffin-embedded specimens were evaluated for the cases diagnosed in 2006 and 2007 (cases 3 and 4), and an ethanol-fixed specimen was evaluated for the case diagnosed in 2009 (case 5). No specimen was available from the 1982 case (case 1) because of the sociopolitical crisis that occurred around 1990.

A DNA tissue kit (Qiagen, Hilden, Germany) was used for extracting DNA from each specimen. For paraffin-embedded specimens, histological sections were processed using xylene and ethanol for paraffin removal and were then rehydrated before DNA extraction. Because of the degradation of DNA, it is difficult to obtain long-fragment DNA from formalin-fixed tissues. Thus, for cases 2–4, we performed polymerase chain reaction (PCR) using primer pairs, which can amplify small

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FIGURE 1. Histopathological pictures of AE case 4 showing (A) characteristic laminated layers of *E. multilocularis* (Periodic Acid Schiff [PAS] staining), (B) active outer lesion with germinal cells and germinal layer (Hematoxylin staining), and (C) inactive or necrotic inner lesion without germinal cell or germinal layer but PAS-positive laminated layers (PAS staining). This figure appears in color at www.ajtmh.org.

(100–200 bp) fragments of *cox1*. For case 5, complete *cox1* was amplified. Some of the primers were already reported elsewhere,¹⁷ and others were newly designed for the present study (Table 1).

All PCRs were performed in 20-µL volumes containing 0.5 units of Ex Taq Hot Start Version (TaKaRa, Ohtsu, Japan), 0.2 mM of dNTP, 1 × Ex Taq Buffer with a final MgCl₂ concentration of 2.0 mM, 15 pmol of each primer, and 1.0 µL of genomic DNA. PCR amplification consisted of initial denaturation at 95°C for 2 minutes, 35 cycles of 95°C for 15 seconds, 53°C for 15 seconds, and 72°C for 20 seconds, and a terminal extension at 72°C for 1 minute. PCR products were directly sequenced, and the obtained sequences were concatenated. Partial *cox1* sequences (1,543 bp) from cases 3 and 4 and a complete *cox1* sequence (1,608 bp) from case 5 were obtained. To estimate the genealogical relationship among the haplotypes in the world, the statistical parsimony network of *cox1* haplotypes was constructed by TCS 1.21.¹⁸ Mitochondrial DNA studies have already revealed that isolates of *E. multilocularis* occur in the Northern Hemisphere, and they are divided

FIGURE 2. Immunoblot and immunochromatographic figures of the two AE cases in Mongolia using a recombinant Em18.^{11,12} (A) Immunoblot figures. Lane a, shows a pooled negative control serum in 1:50 dilution, lane b, shows confirmed AE serum in 1:1000 dilution, and lanes c and d show sera from cases 4 and 5, respectively, in 1:20 dilution. (B and C) Immunochromatographic figures of cases 4 and 5, respectively.¹⁰

into four clades: European, Asian, North American, and Inner Mongolian.¹⁷ Case 4 was of the “Asian” type, whereas cases 3 and 5 were of the “Inner Mongolian” type (Figure 3). Case 2 was also confirmed as AE based on *cox1* sequence, but it was not included in the haplotype network analysis because of the short sequence (659 bp).

All AE cases confirmed from the western parts of Mongolia, which are located between Russia and China where highly endemic AE foci were previously described by WHO,¹ had no history of going abroad and were concluded to be indigenous AE cases. This leads to the hypothesis that there may be one large focus of AE in the mountainous region that includes parts of Mongolia, China, and Russia.

The coexistence of the Asian and North American haplotypes was reported from the St. Lawrence Island in the Bering Sea, and an evolutionary scenario in which distinct parasite populations derived from glacial refugia have been maintained by indigenous host mammals was discussed.¹⁷ The present results indicate that human AE cases in Mongolia show additional and different coexistence of the Asian and Inner Mongolian haplotypes. Therefore, further studies on the genetic diversities of the parasite through identification of the natural intermediate and definitive host animals in Mongolia are necessary and essential to discuss the evolution of this parasite.^{1,17} Surveillance of *E. multilocularis* in humans and animals, such as rodents (intermediate hosts), red foxes (*Vulpes vulpes*), corsac foxes (*Vulpes corsac*),¹⁹ wolves, and dogs (definitive hosts), is currently under discussion between the National Center for Communicable Diseases in Mongolia and Asahikawa Medical College in Japan.

Received September 4, 2009. Accepted for publication October 26, 2009.

Acknowledgment: We thank Dr. Christine Budke for editing this manuscript.

FIGURE 3. Haplotype network constructed with the program TCS 1.21¹⁸ based on 1543 base pairs of *cox1* sequences.¹⁷ Each node indicates a mutational change, and the geographic names inside circles represent the origins of haplotypes. GenBank accession numbers of each sequence used in the present study are as follows: Austria (AB461412), France (AB461413), Slovakia (AB461414), Kazakhstan (AB461415), Japan (AB461416), China 1 (AB461417), China 2 (AB477010), China 3 (AB477011), China 4 (AB477012), USA 1 (AB461418), USA 2 (AB461419), China 5 (AB461420), Mongol 2 (AB510022), Mongol 3 (AB510023), Mongol 4 (AB510024), and Mongol 5 (AB510025).

Financial support: This study was supported in part by a Grant-in-Aid for international research projects on cestode zoonoses in Asia (17256002, 21256003) and by the Asia-Africa Science Platform Fund (2006-2011) from the Japan Society for the Promotion of Science (to A.I.). N.M. received a World Health Organization/Special Programme for Research and Training in Tropical Diseases (WHO/TDR) Grant (200075162). Nucleotide sequence data reported in this paper are available in the DNA Data Bank of Japan/GenBank/European Molecular

Biology Laboratory (DDBJ/GenBank/EMBL) databases under accession numbers AB510022 (Mongol 2), AB510023 (Mongol 3), AB510024 (Mongol 4), and AB510025 (Mongol 5).

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TABLE 1
Primers used for the amplification of *cox1* gene fragments of *E. multilocularis*

Primers	Sequence (5' to 3')	Positions	Reference
Em <i>cox1</i> F1	gactttctcttgggtggtgaag	tRNA-ser	17
Em <i>cox1</i> F11	tgtgattagtagtgggtattga	11–34	PS
Em <i>cox1</i> F100	ttaagtttagttgtgattcgtg	100–124	PS
Em <i>cox1</i> F9	tgactaatcatggtataataatgac	182–207	PS
Em <i>cox1</i> F400	tcttctcatattttctaggagtag	400–425	PS
Em <i>cox1</i> F500	gtactttgtatagtgtttatgact	500–525	PS
Em <i>cox1</i> F4	gttttggctgctgctattactatgc	601–625	PS
Em <i>cox1</i> F2	gtttatgttttattctgctggatt	727–752	PS
Em <i>cox1</i> F5	tgggttggaatggaagacggcgt	885–908	PS
Em <i>cox1</i> F1000	aagagtgcctatttttgggtgggt	1000–1025	PS
Em <i>cox1</i> F1100	ttttacacgatactgtatttgggtg	1100–1125	PS
Em <i>cox1</i> F6	attactggttgaggtgaataagt	1201–1225	PS
Em <i>cox1</i> F10	cctatgcattatttgggttatgtg	1282–1306	PS
Em <i>cox1</i> F7	tctttatatctgcgttagtggtg	1375–1400	PS
Em <i>cox1</i> R1	aacctaaacaacacttcacag	<i>rml</i>	17
Em <i>cox1</i> R11	gtaattatgtatttctgttggtag	1583–1608	PS
Em <i>cox1</i> R10	tagtggaattattgaagtcctgta	1475–1500	PS
Em <i>cox1</i> R1400	cttttatatctgcgttttagtggtg	1376–1400	PS
Em <i>cox1</i> R9	tatgtgggtgacctgctgtgtg	1301–1325	PS
Em <i>cox1</i> R1200	ttatgtttattgatggtgacctgt	1175–1200	PS
Em <i>cox1</i> R4	cgatactgtatttgggtgctcat	1107–1131	PS
Em <i>cox1</i> R8	atgttgcttaattctagtgaata	976–1000	PS
Em <i>cox1</i> R7	gtttgggttttatggtttgttt	801–825	PS
Em <i>cox1</i> R2	ttcagcatatgttttgggttttgg	692–716	PS
Em <i>cox1</i> R600	tattttattgttagtgacctgct	576–600	PS
Em <i>cox1</i> R400	tgggttgacttttatctccattgt	375–400	PS
Em <i>cox1</i> R12	gggtgtttgtctgattgaattgcc	268–293	PS
Em <i>cox1</i> R200	tttttgggtgactaatcatggtataat	175–200	PS

PS = present study; tRNA-ser = tRNA gene for serine; *rml* = large subunit ribosomal RNA.

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