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Short Report: Genetic Analysis of *Echinococcus multilocularis* Originating from a Patient with Alveolar Echinococcosis Occurring in Minnesota in 1977

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Abstract. To date, only a single proven case of autochthonous human alveolar echinococcosis has been recorded in Minnesota in 1977. At that time, echinococcal lesions removed from the patient were experimentally inoculated into voles, and the parasite materials obtained from the voles were preserved as histopathologic specimens for 30 years. In this study, retrospective genetic analysis of larval *Echinococcus multilocularis* originating in the human case was performed using the histopathologic specimens. DNA was extracted from the hematoxylin and eosin–stained specimens, and mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) was amplified by polymerase chain reaction. Subsequently, 20 small fragments (100~216 bp) covering almost the entire sequences (97%) of the *cox1* were successfully amplified, and the nucleotide sequence analysis showed that the *E. multilocularis* isolate from Minnesota was almost identical to an isolate from South Dakota rather than isolates from contiguous Alaska.

Alveolar echinococcosis (AE) in humans is a potentially lethal parasitic disease caused by the larval stage (metacestode) of the fox tapeworm, *Echinococcus multilocularis* and occurs in most of the northern hemisphere, including central Europe, most of northern and central Eurasia, and parts of North America.[1] The metacestode proliferates like a tumor in various organs, mainly liver, and develops into multivesiculated lesions. Clinical symptoms such as jaundice, epigastric pain, fatigue, and/or hepatomegaly may appear after an asymptomatic period of 5–15 years, and treatment may be difficult despite a variety of surgical and chemotherapeutic approaches.[2]

In North America, *E. multilocularis* is distributed in two distinct geographic regions: the northern tundra zone (western Alaska, Arctic islands such as St. Lawrence Island, and Canadian Arctic Archipelago), and northern central America, including three provinces of Canada and 13 contiguous states of the United States.[1,3,4] In Alaska, the tapeworm infections are perpetuated in a sylvatic cycle with carnivores, mainly arctic foxes (*Alopex lagopus*), red foxes (*Vulpes vulpes*) as definitive hosts, and small rodents such as voles (*Microtus oeconomus*) and brown lemming (*Lemmus trimucronatus*) as intermediate hosts.[3] Domestic dogs can also harbor the tapeworm. In central North America, red foxes, grey foxes (*Urocyon cinereoargenteus*), and coyotes (*Canis latrans*) as definitive hosts and red-backed voles (*Clethrionomys rutilus*), meadow voles (*Microtus pennsylvanicus*), bushy-tailed woodrat (*Neotoma cinerea*), and deer mouse (*Peromyscus maniculatus*) as intermediate hosts are involved in the completion of the parasitic cycle.[4]

Regarding human AE in North America, 73 cases were reported between 1951 and 1993: 71 were in Alaskan Eskimos,[5] and 2 cases were from Winnipeg, Manitoba, Canada in 1937[6] and Minnesota in 1977.[7,8] Regarding genotypes of *E. multilocularis* isolated from humans, there is no information about genotypes of *E. multilocularis* from the United States, although a few isolates from Canada have been genetically examined.[9,10]

In this study, to examine the genotype of *E. multilocularis* originating from the AE patient reported in Minnesota in 1977,[7] mitochondrial DNA analysis was performed using archival specimens prepared at that time. The case was autochthonous to Minnesota, and the patient was a 56-year-old woman. The patient complained of epigastric discomfort and malaise and was first suspected to have a malignancy. However, the clinical, serologic, and pathologic findings led to the diagnosis of AE. In addition, the identification of *E. multilocularis* was confirmed by metacestodes developed in red-backed voles (*C. rutilus*) inoculated intraperitoneally with tissue from the hepatic lesions of the patient. The parasite materials obtained from the voles were processed for histopathology and were preserved as hematoxylin and eosin (HE)-stained specimens for the past 30 years.

Template DNAs for polymerase chain reaction (PCR) were prepared by the method described previously[11]: the HE-stained sections were rinsed in xylene after removal of coverslips, washed in absolute ethanol, and air dried. At first, 10 μL of 0.05 N NaOH solution was placed onto some sections, and the sections were scalped, collected into Eppendorf tubes, and heated at 95°C for 1 hour. As an alternative method, a DNA Isolator PS kit (Wako Pure Chemicals, Osaka, Japan) was used for DNA extraction from the remaining sections. The PCR amplification of the cytochrome *c* oxidase subunit 1 gene (*cox1*) was performed in a 50-μL reaction mixture as reported previously.[12] Primer pairs used are shown in Table 1. F17 was used in combination with both R17 and R18. F20 was used with R18. The reaction was performed for 35 cycles of denaturation (94°C, 30 seconds), annealing (58°C, 30 seconds), and extension (72°C, 90 seconds) with a thermal cycler (GeneAmp 9700; PE Applied Biosystems, Foster City, CA). Direct DNA sequencing for the PCR amplicons was performed using a BigDye Terminator v. 3.1 Cycle Sequencing ready reaction kit (Applied Biosystems). Resultant sequence ladders were read with an ABI PRISM 310 or 3100 Genetic Analyzer (Applied Biosystems).

Because the *E. multilocularis* specimens used were fixed in formalin, and very limited amounts of the sections were available, only 20 small fragments (100~216 bp) were amplified (data not shown). Unfortunately, amplification of two regions using F1/R1 and F3/R3 primer pairs was not successful be-

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TABLE 1
PCR primer pairs used in this study

Primers*	Nucleotide sequences for *cox1*	Positions
F1	ATTTAGGGGCTGGTTGGTCATCTTAT	45–68 for *trnW*
R1	AACCAACAAAACCAGACCATA	100–80
F2	ATGAGAGTGGTGTGATTAGGTAG	1–23
R2	ATTATACCATGATTAGTCACCAAAAA	200–175
F3	TTAAGTTTTAGTTTGTTGATT	100–120
R3†	AATATAGGCATCAAAAAAAAAAA	230–208
F4	TGACTAATCATGGTATAATAATGATC	182–207
R4	GGCAAATTCAAATCAGACAAACCACC	293–268
F5	GTTTGGTAATTATTTATTGCCTTTG	240–264
R5	TGCAAAGAAAACATCAAAAAATCAA	455–431
F6	TGCCACGTTTGAATGCTTTGAGTGCG	290–315
R6	ACAATGGAGGATAAAAAGTCCAACCA	400–375
F7	TCTTCTTCATATTTTTCTAGGAGTAG	400–425
R7	AGGCAACGTCACTAACAATAAAATA	600–576
F8	CTAGAGTTTTTAGTTCTATAAATTT	470–494
R8	CAAAAGCATAGTAATAGCAGCAGCC	630–606
F9	GTACTTTGTATAGTGTTTTTATGACT	500–525
R9	CCAAAAAACCAAAACATATGCTGAA	716–692
F10	CGTTAGGTGGTGGTGATCCTATTCTA	665–690
R10	ACTCCCTAAACACACTATAGAAAACA	855–830
F11	ATGTTTTGGTTTTTTGGTCATCCGGA	700–725
R11	AAACAACAAACCATAAAACCCAAAC	825–801
F12	CGTTTGGGTTTTATGGTTTGTTGTTT	800–825
R12	TATTTACACTAGAATTAAGCAACAT	1,000–976
F13	GACTGGTATAAAGGTGTTTACTTGGT	945–970
R13	CACCACCAAACGTAAACAACACTAT	1,060–1,036
F14	AAGAGTGATCCTATTTTGTGGTGGGT	1,000–1,025
R14	CAACGGTCACCATCAAATAAACATAA	1200–1175
F15	TGTTATGTCGTTAGGTTCTTATATAA	1,140–1,165
R15	AATATTAGAAATTATACACTGACAT	1,260–1,236
F16	ATTACTGGTTTGAGGTTGAATAAGT	1,201–1,225
R16	CACCCACTAAACGCAGATATAAAAG	1,400–1,376
F17	ATTGGGTTAAAATGGTTTGTACTGT	1,346–1,370
R17	AGACCTCTTTCTTACTTACCATAGA	1,450–1,426
R18	CACCATAAGTATAATCAACACTATA	1,555–1,531
F19	TCTTTTATATCTGCGTTTAGTGGGTG	1,375–1,400
R19	TACAGGACTCATTAAATAATCCACTA	1,500–1,475
F20	GAAAGAGGTGTTGGGTTCATATAAA	1,440–1,464
F21	AGCTTGTCATAATGATTATTTTTGTT	1,500–1,525
R21	CTAACCAACAGCAAATACATAATTAC	1,608–1,583
F22	GATTATACTTATGGTGTATATTATAT	1,540–1,565
R22	ATCATAAACTTTAACTAACTAACC	24–1 for *trnT*

* Forward (F) and reverse (R) primers having the same number were basically used as primer pairs.
† Designed based on AB353729. Other primers from AB018440.

cause of the lack of DNA sample and positions 1–23 and 175–207 were not determined. The total number of nucleotide sites determined from the 20 *cox1* fragments was 1,552. Of these sites, 11 were substituted compared with known *cox1* sequences of *E. multilocularis* isolates (Table 2). The nucleotide sequences between two isolates from Minnesota and South Dakota were the same except for a nucleotide at position 688: C for Minnesota and T for South Dakota (Table 2). There were nine transitional substitutions between isolates from Minnesota and Alaska. Higher sequence homologies were shown with *E. multilocularis* from South Dakota (99.9%) compared with *E. multilocularis* from contiguous Alaska and from Japan (99.4%), indicating that *E. multilocularis* isolates from Minnesota and South Dakota belong to the same genotypic group.

There are some reports on genotypes of *E. multilocularis* isolates from Alaska and contiguous Canada and the United States; however, the number of *E. multilocularis* genotypes is variable (1–4) depending on the target genes and number of specimens examined.[9,10,13,14] The Minnesota isolate reported here seems to be identical to the genotype from Montana and Canada based on geographic locations.

Despite the widespread occurrence and high prevalence of *E. multilocularis* infections in definitive hosts in North America,[4,15–17] human cases seem to be few compared with the number of AE patients reported from Japan[18] and Europe,[19–21] where the prevalence of the *E. multilocularis* infection in definitive hosts is also high. In the extensive Arctic and sub-Arctic regions of Canada where *E. multilocularis* is endemic in definitive host animals,[4] cases of human AE have never been reported. To date only two cases of AE were diagnosed in the contiguous north-central region of Canada and the United States.[6,7] The reasons are not fully understood; however, human behavioral factors[21] and life cycles of the parasite involving synanthropic or sylvatic transmission[4,18] may be hypothesized. Genetic variations[9,10,13,14] and biological attributes[22] among *E. multilocularis* isolates and immunogenetic factors in humans[23–28] might play some roles in infectivity of *E. multilocularis* to humans. Because there are limited data on genotypes/haplotypes of *E. multilocularis* from North America except for Alaska, the data reported here may also provide useful information in considering the phylogeography of *E. multilocularis*.[29,30]

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TABLE 2
Base-substituted sites in cytochrome *c* oxidase subunit 1 genes among six *E. multilocularis* isolates

E. multilocularis isolates from	Accession numbers	Base-substituted sites										
		289	585	688	735	760	800	822	1,314	1,329	1,351	1,573
Minnesota	AB353729	C	A	C	G	G	T	G	G	A	A	G
South Dakota*	AB374425	C	A	T	G	G	T	G	G	A	A	G
Alaska*	Same as AB018440	T	G	C	T	A	C	G	T	G	G	A
Hokkaido, Japan	AB018440	T	G	C	T	A	C	G	T	G	G	A
Fukui, Japan†	AB385610	T	G	C	T	A	C	G	T	G	G	A
Slovak Republic	DQ013305	‡	‡	‡	‡	A	C	A	‡	‡	‡	‡

* The isolates from South Dakota and Alaska were derived from fox and vole, respectively (Nakao and others, unpublished data).
† This case was diagnosed at autopsy in a remote area from Hokkaido, Japan.[11]
‡ Data not available.

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