# Supplementary Appendix

Supplement to: Polish LB, O'Connell EM, Barth TFE, et al. European haplotype of *Echinococcus multilocularis* in the United States. N Engl J Med 2022;387:1902-4. DOI: 10.1056/NEJMc2210000

This appendix has been provided by the authors to give readers additional information about the work.

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*Echinococcus multilocularis* in the United States: Evidence for a changing epidemiology and local transmission of the European Haplotype

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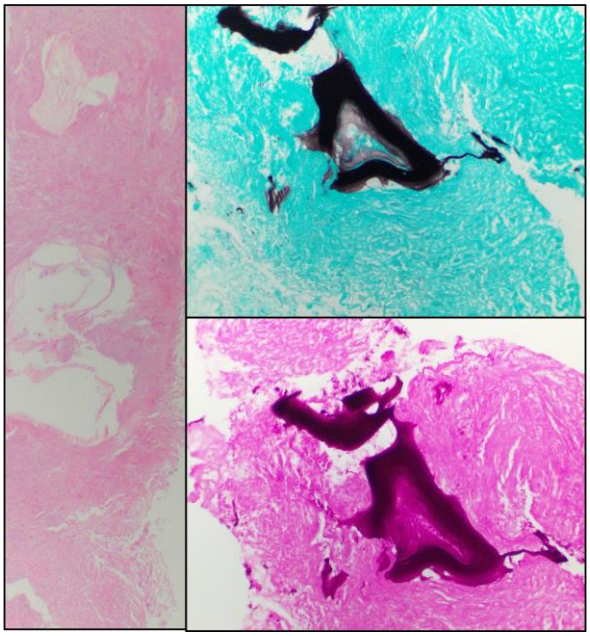
**Additional History:**

The patient had a mild dementia with some memory loss but was otherwise fully cognizant. Both he and his wife provided a detailed history of travel which included the following: He was born in New York City, had lived in Ohio, Pittsburgh, Wilkes Barre, Pennsylvania and in Vermont for the past 25 years. He has had dogs his entire life. He does hike in the woods. His travel has included a junior year in Paris in 1957 with some traveling around France. In 1961 was in the French Congo for 10 weeks at Crossroads Africa similar to the Peace Corps, 1964 for approximately 10 days was in England, Scotland and Ireland. In 1979 spent a couple of weeks on safari in Kenya. In 1986 Austria and Switzerland hiking mountains, train to Vienna, Mexico in 2005 and in 2015 spent approximately 2 weeks in Paris and the Loire Valley. He has not had any other fox or animal exposures. Although in the distant past he had been to countries where *E. multilocularis* is known to be found, given the timing and duration of his travel as well as his exposure history, acquisition of infection from these trips was felt to be highly unlikely.

There have been limitations at this time assessing fox populations in Vermont and Maryland, although this work is being pursued. Foxes were screened in Virginia due to availability. VT pt #1 grew up in Maryland, very close to the area in Norther Virginia where these foxes were found. We are not purporting that we have identified the fox population that served as the host for the strains of Em that our patients acquired, but the fact that this strain--so genetically close to what was found in these patients--was found in foxes in regions not historically endemic for Em makes it likely that this particular strain has a wide area of geographic distribution in the US, particularly since VT pt #2 has not spent much time in the Maryland/Virginia area. The full extent of its distribution deserves further investigation, and is an area of interest to the authors. Since *E. multilocularis* eggs need to be stored at –80C for at least five days in order to be inactivated and we do not have enough space at -80C, we only requested fox fecal samples from hunters, trappers, and some wildlife rehabilitators. Therefore, foxes were not available for searching adult tapeworms.

**Liver Biopsy:**



SFigure1: Left panel: Core biopsy composed of dense fibrosis and hyaline necrotic tissue with embedded refractile parasite fragments (H&E, 20x); Top right panel: Densely Ammonical silver staining of the refractile parasite walls (Ammonical Silver, 100x); Lower right panel: Periodic acid Schiff positive staining parasite walls (PAS, 200x)

**Materials and Methods:**

Fecal samples of 296 foxes and 138 coyotes from Virginia counties concentrated in the northern and northwestern portions of the state were collected by the Virginia Department of Wildlife Resources. Samples were provided by hunters and trappers. A small number of samples came from wildlife rehabilitators and road killed animals. Fecal samples were submitted to IDEXX Laboratories and were tested with the commercial qPCR test for *Echinococcus* spp. and *Echinococcus multilocularis*. Two red foxes (identified as 146 and 197) from Northern Virginia, Clarke and Loudoun counties, tested positive for *E. multilocularis*.

*E. multilocularis*-positive fecal samples of two red foxes (identified as 146 and 197) were analyzed. *E. multilocularis* infection was confirmed by amplification of a 395-bp fragment of the mitochondrial gene encoding the NADH dehydrogenase subunit 1 (NAD1) by PCR using the *E. multilocularis* specific primers Cest1 and Cest2 (1). For genotyping, PCR amplification of complete mitochondrial NADH dehydrogenase subunit 2 (NAD2) and cytochrome b (COB) using published primers (2) was performed for both foxes samples and for the human patient (VT#1) by Polish et al (3). COB primers did not produce the expected amplicon, so a partial fragment of COB gene (nt 209-902) was amplified using the set of primers previously published (4). The PCR was carried out in a 50 µl reaction mixture using Platinum PCR SuperMix High Fidelity (Invitrogen), with an initial denaturation step at 94°C for 1 minute, 35 amplification cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 1 minute, and a terminal extension step at 68°C for five minutes. PCR products were purified using the QIAquick PCR purification kit (Qiagen) and Sanger sequenced in both the forward and reverse directions. The sequences obtained were aligned using Geneious Prime 2021 (https://geneious.com) and compared to the reference sequences from the GenBank database. The consensus sequences of NAD2 and COB for foxes and the VT#1 patient are available in the GenBank database under the accession numbers OK268248, OK268249, OK268250, OK268251, OK268252, and OK268253.

COX1 amplification was performed with previously described primers (2) Q5®High-Fidelity DNA Polymerase (New England Biolabs, Cat No. M0491S) per manufacturer's instructions with the following thermocycling conditions: Initial denaturation 30 seconds at 98°C , followed by 40 cycles of 98°C for 5 seconds, 30 seconds of annealing (at 60, 61, 62, and 63°C in parallel), 60 second extension 72°C, and final extension for 2 minutes at 72°C. High sensitivity Bioanalyzer analysis (Agilent) demonstrated peaks ~2000 bp on samples subjected to an annealing temperature of 63°C. DNA concentration at 2000 bp from fox 197 was <1ng/µl, therefore 1 µl of DNA was subjected to nested PCR with select primers from Yamasaki et al (5). The nested products were combined with the previously obtained PCR products for sequencing. DNA from each fox was sequenced separately by Oxford Nanopore sequencing (Oxford Nanopore Technologies, FLO-MIN111 R10.3v) using the SQK-LSK109 protocol. Sequencing was run until completion. Guppy (Oxford Nanopore) was utilized to convert fast5 to FASTQ files. Geneious Prime 2021 (https://geneious.com) was used to align and trim reads to a reference *E. multilocularis* COX1 sequence (GenBank AB461412). The consensus sequence (1500X coverage for fox 146, 5000X coverage for fox 197) were deposited on GenBank (OK330092 and OK330093, respectively).

**Phylogenetic tree construction:**

Sequences obtained from this paper, as well as all *E. multilocularis* COB sequences over 665 base pairs (bp), all COX1 sequences over 1604 bp, and all NAD2 sequences over 875 bp were downloaded from NCBI on September 3, 2021, using MegAlign Pro v17.1.1. Out of these files, those isolates that had NAD2, COB, and COX1 sequences in GenBank were correlated based on available literature, representative sequences chosen to represent the broad range of haplotypes, and sequences were trimmed, concatenated, aligned (Clustal Omega), and phylogenetic trees constructed in Geneious Prime 2021 using maximum likelihood with bootstrap analysis with 1000 iterations.

**VT Patient #2 Targeted SNP sequencing**

Twenty (20) unstained slides from a formalin-fixed, paraffin embedded (FFPE) liver biopsy sample from VT#2 patient underwent DNA extraction using QIAamp DNA FFPE Tissue Kit (Qiagen) per manufacturer's instructions, and short fragments of the COX1 gene were amplified, cloned, and underwent Sanger sequencing as previously described[3]. Due to the fragmented nature of the fixed DNA, the entire COX1 gene could not be sequenced with this approach, so additional small fragment SNP-directed COX1 and COB-targeted primers were designed (STable1). Successfully amplified fragments were cloned and sequenced.

**Table S1: COX1, COB & NAD2 Sequences**

| Country | NAD2 | COB | COX | |
|---|---|---|---|---|
| Japan | AB461407 | AB461399 | AB461416 | |
| Austria | AB461403 | AB461395 | AB461412 | |
| France | AB461404 | AB461396 | AB461413 | |
| Slovakia | AB461405 | AB461397 | AB461414 | |
| Kazakhstan | AB461406 | AB461398 | AB461415 | |
| China: sichuan | AB461408 | AB477009 | AB477010 | |
| USA_AK | AB461409 | AB461400 | AB461418 | |
| USA_IN | AB461410 | AB461401 | AB461419 | |
| Mongolia | AB461411 | AB461402 | AB510025 | |
| Canada (BC) | KC550005 | KC550003 | KC550004 | |
| Poland | KY205692 | KY205662 | KY205677 | Pol1 |
| Poland | KY205693 | KY205663 | KY205678 | Pol2 |
| Poland | KY205694 | KY205664 | KY205679 | Pol3 |
| Kyrgyzstan | MN829513 | MN829497 | MN829529 | A11 |
| Kyrgyzstan | MN829514 | MN829498 | MN829530 | A12 |
| Kyrgyzstan | MN829515 | MN829499 | MN829531 | A13 |
| USAVA (fox) | OK268248 | OK268250 | OK330092 | this paper |
| USAVA (fox) | OK268249 | OK268251 | OK330093 | this paper |
| USAVT (human) | OK268252 | OK268253 | MN387224 | this paper and previously published |

**TableS1: List of all COX1, COB, and NAD2 sequences concatenated for phylogenetic tree in Figure 1.**

**Table S2: COX 1 and COB Primer Sequences**

| Name | Primer type | Sequence | Nucleotide start | Primer length | Successful? |
|---|---|---|---|---|---|
| COB 1 | Forward Primer | GGGTGGCACTAGTGTTTATAG | 894 | 21 | |
| COB 1 | Reverse Primer | GGTGACACCCACCTAAATAAG | 988 | 21 | No |
| COB 2 | Forward Primer | TAGTTATGGGTGAGGCATTTAC | 338 | 22 | |
| COB 2 | Reverse Primer | ACCAACTAACGGCAAACTAT | 444 | 20 | Yes |
| COB 13 | Forward Primer | GTGGTAACAGTAATCCGTTGT | 581 | 21 | |
| COB 13 | Reverse Primer | CAGCCTCTAAATATGCCTCTATATC | 754 | 25 | Yes |
| COB 23 | Forward Primer | ACTGGAGTATTGTTGTCTTT | 112 | 20 | |
| COB 23 | Reverse Primer | CAGTACATTTACACCTACCATA | 243 | 22 | Yes |
| COX 2 | Forward Primer | ATTCTGCCTGGATTTGGTATAA | 739 | 22 | |
| COX 2 | Reverse Primer | CCCAAACACTACTCCCTAAAC | 865 | 21 | No |
| COX 13 | Forward Primer | TTTGGCTGCTGCTATTACTAT | 603 | 21 | |
| COX 13 | Reverse Primer | AACATAAACCTCCGGATGAC | 735 | 20 | No |
| COX 30 | Forward Primer | GATCATAAGCGTATTGGAGTGA | 40 | 22 | |
| COX 30 | Reverse Primer | CACCCAACAAAGGCAATAAAT | 271 | 21 | Yes |

**TableS2: Primer sequences directed at amplifying short DNA fragments across COX1 and COB genes for *Echinococcus multilocularis,* used on DNA extracted from FFPE tissue from VT Patient #2. Not all primer pairs were successful at producing amplification, as indicated.**

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