

Noninvasive Detection of *Echinococcus multilocularis* Tapeworm in Urban Area, Estonia

Leidi Laurimaa, John Davison, Liivi Plumer, Karmen Süld, Ragne Oja, Epp Moks, Marju Keis, Maris Hindrikson, Liina Kinkar, Teivi Laurimäe, Jaana Abner, Jaanus Remm, Peeter Anijalg, and Urmas Saarma

Author affiliation: University of Tartu, Tartu, Estonia

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To the Editor: Alveolar echinococcosis, which is caused by the fox tapeworm *Echinococcus multilocularis*, is an emerging disease in Europe that shows a high mortality rate (1). Humans can become infected after ingesting parasite eggs (e.g., through direct contact with dogs and red foxes [*Vulpes vulpes*] or with their contaminated feces). *E. multilocularis* tapeworm eggs are extremely resistant and can remain viable in the environment for years (2).

Numbers of red foxes have increased in many countries in Europe in recent decades, and the *E. multilocularis* tapeworm has also expanded its range. This tapeworm has recently been reported in 17 countries in Europe, including Lithuania, Latvia, and Estonia (1). Foxes and associated tapeworms are also increasingly found in urban areas, prompting considerable public health concern (1,3). Foxes began to colonize urban areas in Estonia in 2005, and they have since been reported in 33 of 47 towns nationwide (L. Plumer et al. unpub. data). Because $\approx 30\%$ of foxes are

infected with the *E. multilocularis* tapeworm in natural habitats in Estonia (4), it is essential to monitor parasite spillover into urban areas, where it could become a serious public health risk. Consequently, there is an acute need for methods that can effectively detect the parasite and thereby help prevent human infection.

Although immunologic (2) and genetic methods (5–7) are available for identifying *Echinococcus* spp. parasites, a sensitive molecular diagnostic method that detects tapeworms and identifies their host species from degraded fecal samples would be useful. The purposes of this study were to develop a sensitive, noninvasive, genetic method to identify the host species by discriminating between feces of red foxes and dogs; detect *E. multilocularis* tapeworms in feces and distinguish them from the related parasite *E. granulosus*; and collect carnivore feces in an urban area in Estonia to identify this tapeworm.

Fecal samples suspected to be from red foxes were collected during January–March 2012 and January–March 2013 from streets and grassy areas of Tartu, Estonia. Tartu is a relatively small city (area 39 km²) with 98,000 human inhabitants. We surveyed 14 transects, each ≈ 4 km in length, that included all major districts in the city (Figure). Each transect was searched weekly during the study period (total ≈ 850 km surveyed).

A total of 137 fecal samples were collected and stored at -80°C for ≥ 1 week to avoid risk of infection from any *Echinococcus* spp. eggs present (2) because *E. multilocularis* (4) and *E. granulosus* (8,9) tapeworms have been found in Estonia. Samples of ≈ 250 mg were placed into 2-mL tubes, heated at 65°C for 15 min, and stored at -80°C . The heating and cooling procedure helps to break the parasite egg shells, enabling more efficient DNA

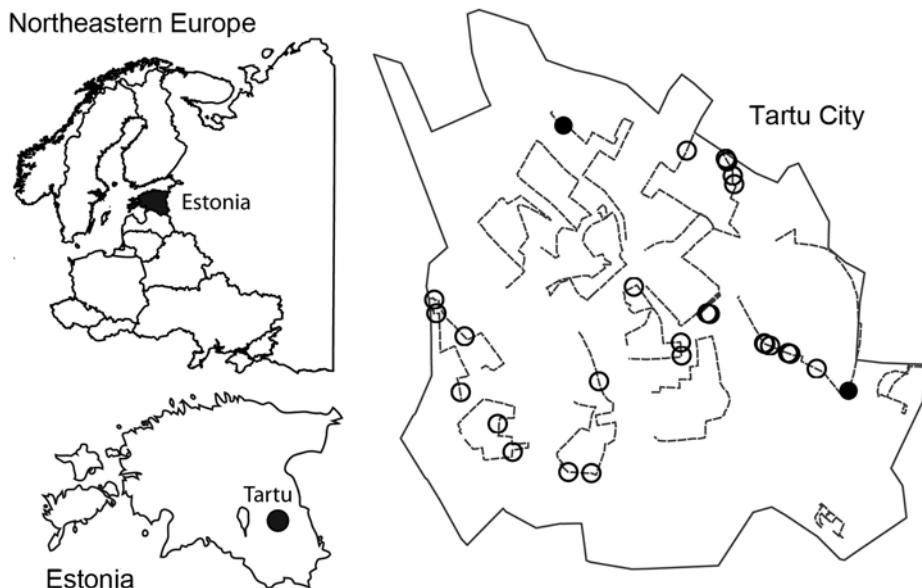


Figure. Location of Tartu in northeastern Europe, Estonia, and red fox feces sampling area in Tartu. The Tartu City boundary is indicated by a solid black line, survey transects are indicated by dashed lines, and fox fecal samples ($n = 28$) are indicated by circles. Filled circles ($n = 2$) indicate samples positive for *Echinococcus multilocularis* tapeworms.

extraction. DNA was extracted by using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Species-specific primers (online Technical Appendix Table, <http://wwwnc.cdc.gov/EID/article/21/1/14-0136-Techapp1.pdf>) were designed to amplify short sequences of mitochondrial DNA. On the basis of primer specificity and amplicon size, we determined host and parasite species (online Technical Appendix Figure 1). DNA extraction and PCR were performed in a laboratory dedicated to environmental samples (for a complete description of methods, see online Technical Appendix).

DNA was successfully extracted and amplified from 119 (86.9%) of 137 fecal samples. Of usable samples, 28 (23.5%) were from red foxes and 91 (76.5%) were from dogs. Two fox fecal samples (7.1%; 95% binomial CIs 0.9%–23.5%) were infected with *E. multilocularis* tapeworms; none of the dog samples were infected.

To verify parasite identification, we amplified DNA from the 2 *E. multilocularis*-positive samples with *E. multilocularis*-specific primers and sequenced the amplification products. To verify host species identification, we used primers that produced longer amplification products (327 and 197 bp) than the corresponding PCR primers and sequenced amplification products from 5 fox samples and 5 dog samples. Sequencing procedures were performed according to the methods of Saarma et al. (10).

Sequences from both *E. multilocularis*-positive samples showed 100% identity with an *E. multilocularis* tapeworm sequence (GenBank accession no. AB018440) (online Technical Appendix Figure 2). All sequenced fox and dog samples also belonged to the corresponding species.

To estimate the sensitivity of this noninvasive genetic method, we determined the number of *E. multilocularis* eggs necessary to obtain a positive PCR result (online Technical Appendix Figure 3). One egg was sufficient to give an *E. multilocularis* tapeworm-specific result.

In summary, we developed a noninvasive genetic method that identifies *E. multilocularis* tapeworms and their host species in carnivore fecal samples found in urban environments. Furthermore, these tapeworms can even be detected in fecal samples from red foxes when only 1 parasite egg is present. Thus, this method is highly sensitive and discriminatory and can be used with degraded fecal samples to monitor *E. multilocularis* tapeworms and their hosts.

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Address for correspondence: Urmas Saarma, Institute of Ecology and Earth Sciences, University of Tartu, Vanemuise 46, 51014 Tartu, Estonia; email: urmas.saarma@ut.ee

Severe Delayed Hemolysis Associated with Regulated Parenteral Antimalarial Drug

Julia Lee, Sigmund Kraiden,
Christopher Graham, Andrea K. Boggild,
Katerina Pavenski, Jay S. Keystone,
and Kevin C. Kain

Author affiliations: University of Toronto, Toronto, Ontario, Canada (J. Lee, A.K. Boggild, J.S. Keystone, K.C. Kain); McMaster

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Technical Appendix

Methods Used to Detect *Echinococcus multilocularis* Tapeworm in Red Fox Fecal Samples, Urban Area, Estonia

Methods

DNA Purification from Fecal Samples

Samples were collected and stored at -80°C for ≥ 1 week to avoid the risk of infection with any *Echinococcus* spp. eggs present. Approximately 250 mg was placed into 2-mL tubes, heated at 65°C for 15 min, and stored at -80°C . The heating and cooling procedure helps to break the parasite egg shells, enabling more efficient DNA extraction. DNA was extracted by using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

PCR

Species-specific primers (online Technical Appendix Table) were designed to amplify short sequences of mitochondrial DNA. The *Echinococcus multilocularis* tapeworm-specific primer pair (EMfor1 and EMrev1) amplifies tRNA-Ile/Lys, the *E. granulosus* tapeworm-specific (EGfor1 and EGrev1) and fox-specific (F1 and RVu) primer pairs amplify 12S rRNA, and the dog-specific (Dog1f and HW1r) primer pair amplifies part of the mitochondrial DNA control region. Although the 3 primer pairs were highly specific, the dog primers can potentially amplify gray wolf (*Canis lupus*) DNA. In other similar studies, only the parasite (1) or the host species (2) was detected, and much longer sequences were amplified, making analysis of degraded samples less sensitive. Quantitative real-time PCRs for detection and quantification of *E.*

multilocularis DNA in fox feces are available (3,4) but for many laboratories the cost of these PCRs limits their use.

PCR was performed twice for each sample in a volume of 20 µL containing 10× Advantage 2-SA Buffer and 50× Advantage2-Polymerase Mix (Clontech, Mountain View, CA, USA), 0.2 µmol/L dNTP (Fermentas, Waltham, MA, USA), 0.25 µmol/L of each primer, and 10 µL of purified DNA. The PCR conditions were 95°C for 1 min; 10 cycles at 95°C for 20 s, annealing at 68°C for 20 s (temperature reduced by 0.5°C in each cycle) and extension at 68°C for 30 s; 35 cycles under the same conditions, except that the annealing temperature was 63°C; and a final extension at 68°C for 3 min. PCR products were separated by electrophoresis on 2.5% agarose gels. DNA extraction and PCR was performed in a laboratory dedicated to environmental samples.

Sensitivity Assay for Detecting *E. multilocularis* Tapeworm in Red Fox Feces

To estimate the sensitivity of the noninvasive genetic method, we determined the number of *E. multilocularis* eggs necessary to obtain a positive PCR result. We analyzed 1, 3, 5, 7, 10, 15, and 20 fox tapeworm eggs, which were added to fox fecal samples (≈250 mg) that were previously known to be uninfected with these eggs. The mixture was heated and deep frozen before DNA extraction, and PCR was performed as described above. The assay was repeated three times. We found that 1 egg was sufficient to give an *E. multilocularis* tapeworm-specific result (online Technical Appendix Figure 3). In other studies, flotation methods for concentrating parasite eggs and alkaline lysis of egg shells have been used before PCR analysis (5,6). This study demonstrates that concentrating eggs is unnecessary when applying the method described above.

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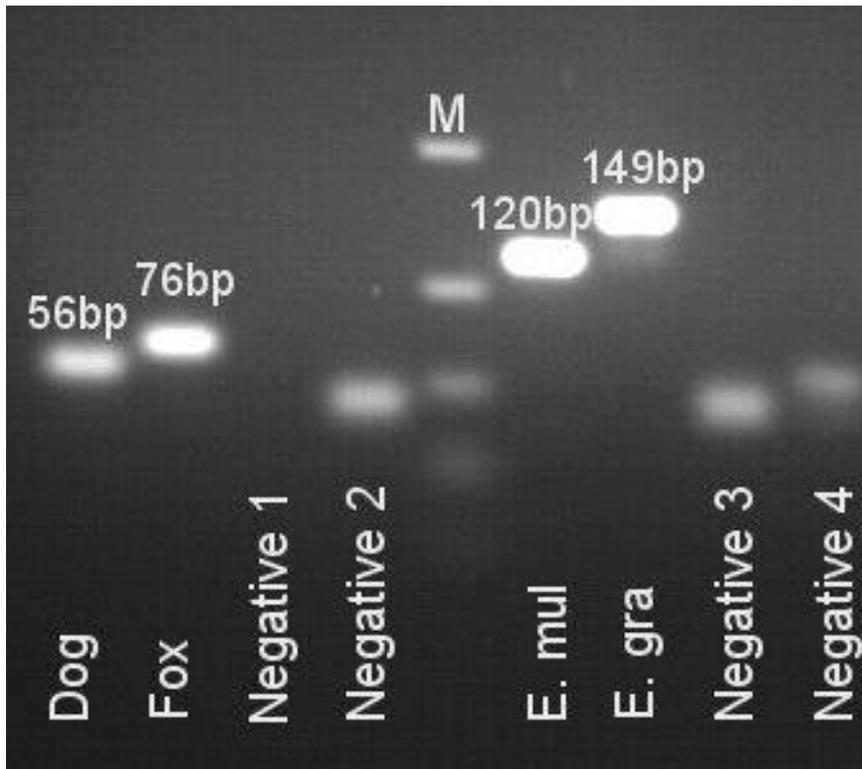
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Technical Appendix Table. Mitochondrial DNA primers used for PCR detection of *Echinococcus multilocularis* fox tapeworm, Estonia

Primer*	Sequence, 5'→3'	Amplicon size, bp	Specificity†
EMfor1	TGATTAATAGGGCTGCATG	120	Fox tapeworm
EMrev1	CACATTACTGAGGTAAGAAC	120	Fox tapeworm
F1	CCATGAAGCACGCACACA	76	Red fox
RVu	GTTAATATGTTTATGGCCTCA	76	Red fox
EGfor1	GTGTGTTACATTAATAAGGGTG	149	Dog tapeworm
EGrev1	CTTGTTACGACTTACCTCAA	149	Dog tapeworm
Dog1f	GTATCTCCAGGTAAACCCTTCTC	56	Domestic dog
HW1r	CAAACCATTAATGCACGACGT	56	Domestic dog

*EM, *Echinococcus multilocularis*; for, forward; EG, *E. granulosus*; rev, reverse.

†Red fox, *Vulpes vulpes*; dog tapeworm, *E. granulosus*; domestic dog, *Canis familiaris*.



Technical Appendix Figure 1. Host and parasite species determination on the basis of primer specificity and amplicon size. PCR amplification of mitochondrial DNA from dog and red fox tissue and from *Echinococcus multilocularis* and *E. granulosus* tapeworm specimens collected in Estonia. Species-specific PCR products for domestic dog (Dog; 56 bp), red fox (Fox; 76 bp), *E. multilocularis* (E. mul; 120 bp), and *E. granulosus* (E. gra; 149 bp) are shown. Lane M, FastRuler Ultra Low Range DNA Ladder (Thermo Scientific, Waltham, MA, USA), DNA fragment sizes are 200, 100 and 50 bp. Lanes Negative 1 and 2, negative controls with no dog or fox DNA added, respectively; lanes Negative 3 and 4, negative controls with no parasite DNA added.

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      10      20      30      40      50      60      70
AB018440  GAA TTTA TTTT CCGT TAA TAT TTTT GACT CAT ATAT CTA ATG TTG CGA AGAG CTG AGT TCT TAC CTA CAG TAA TGT GG
EMest     GAA TTTA TTTT CCGT TAA TAT TTTT GACT CAT ATAT CTA ATG TTG CGA AGAG CTG AGT TCT TAC CTA CAG TAA TGT GG

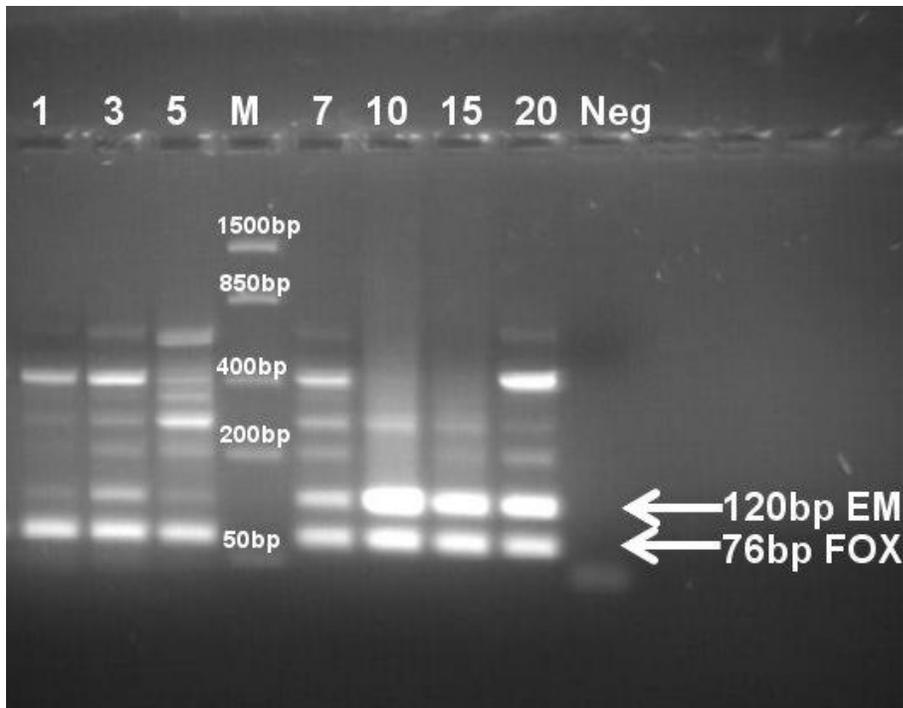
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Sequences producing significant alignments:

Select: [All](#) [None](#) Selected:0

Alignments Download GenBank Graphics Distance tree of results							
	Description	Max score	Total score	Query cover	E value	Ident	Accession
<input type="checkbox"/>	Echinococcus multilocularis mitochondrial DNA, complete genome	141	141	100%	5e-35	100%	AB018440.2
<input type="checkbox"/>	Echinococcus equinus mitochondrion, complete genome	111	111	100%	4e-26	93%	AF346403.1
<input type="checkbox"/>	Echinococcus felidis mitochondrial DNA, complete genome, sample code: EfelUganda	87.9	87.9	100%	6e-19	88%	AB732958.1
<input type="checkbox"/>	Echinococcus granulosus mitochondrial DNA, complete genome, sample code: 52LI07	82.4	82.4	100%	3e-17	87%	AB786664.1
<input type="checkbox"/>	Echinococcus granulosus genotype 1 mitochondrion, complete genome	82.4	82.4	100%	3e-17	87%	AF297617.1

Technical Appendix Figure 2. Nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) results for *Echinococcus multilocularis* tapeworm sequence. Alignment of mitochondrial DNA control region sequences (upper panel). AB018440 is a GenBank sequence for *E. multilocularis* tapeworm. EMest is a homologous sequence of *E. multilocularis* tapeworm from 2 red foxes in Tartu, Estonia, and corresponds to positions 8580–8655 in AB018440. Results from sequence similarity search by Nucleotide BLAST (lower panel). Note that the *E. multilocularis* sequence determined from red foxes in Tartu (EMest) shows 100% identity only with a reference sequence for *E. multilocularis* tapeworm (AB018440). For other *Echinococcus* species, the identity is lower ($\leq 93\%$). Max, maximum; Ident, identity; Accession, GenBank accession number.



Technical Appendix Figure 3. Sensitivity of PCR for detecting *Echinococcus multilocularis* tapeworms in red fox feces collected in Estonia. PCR amplification of mitochondrial DNA fragments from uninfected red fox fecal samples to which various numbers of *E. multilocularis* eggs were added. Lanes 1, 3, 5, 7, 10, 15 and 20, indicate numbers of *E. multilocularis* eggs added to each sample; lane M, FastRuler Low Range DNA Ladder (Thermo Scientific, Waltham, MA, USA). DNA fragment sizes of the ladder are shown in basepairs above the bands; lane Neg, negative control with no parasite eggs added. Arrows indicate PCR products for *Echinococcus multilocularis* (EM; 120 bp) and red fox (FOX; 76 bp).