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HELMINTHOLOGIC SURVEY OF THE WOLF (*CANIS LUPUS*) IN ESTONIA, WITH AN EMPHASIS ON *ECHINOCOCCUS GRANULOSUS*

E. Moks,[1,4] I. Jõgisalu,[1,4] U. Saarma,[1,2,4] H. Talvik,[3] T. Järvis,[3] and H. Valdmann[1,4,5]

[1] Department of Integrative Zoology, University of Tartu, Vanemuise 46, 51014, Tartu, Estonia
[2] Estonian Biocentre, Riia 23, 51010 Tartu, Estonia
[3] Department of Infectious Diseases, Estonian Agricultural University, Kreutzwaldi 62, 51014, Tartu, Estonia
[4] Authors contributed equally to this work
[5] Corresponding author (email: Harri.Valdmann@ut.ee)

ABSTRACT: Carcasses of 26 wolves were collected during the 2000/2001 and 2003/2004 hunting seasons and examined for helminths. Thirteen helminth species were recorded: one trematode (*Alaria alata*), seven cestodes (*Diphyllobothrium latum*, *Mesocestoides lineatus*, *Taenia hydatigena*, *Taenia multiceps*, *Taenia ovis*, *Taenia pisiformis*, and *Echinococcus granulosus*), and five nematode species (*Uncinaria stenocephala*, *Toxascaris leonina*, *Toxocara canis*, *Trichinella nativa*, and *Trichinella britovi*). The most common species were *A. alata* and *U. stenocephala*. Mature *Echinococcus granulosus* was found and described for the first time in Estonia, and its identity verified using PCR-RFLP analysis. Sequencing a fragment of the mitochondrial DNA NADH dehydrogenase 1 (mtND1) gene showed that the *E. granulosus* strain from Estonia was identical to strain G10, recently characterized in reindeer and moose in Finland.

Key words: *Canis lupus*, *Echinococcus granulosus*, Estonia helminths, *Trichinella*.

INTRODUCTION

Wolves (*Canis lupus*) have been present in Estonia since the last Ice Age, and in some periods they have been numerous (Lepiksaar, 1986; Lõugas and Maldre, 2000). During recent centuries, the wolf has been managed extensively and its numbers have fluctuated depending on hunting pressure and immigration (Valdmann et al., 2004a). Close association with the Russian wolf population has been a key factor for the abundance of the Estonia wolf population, no matter how extensive the hunting. According to 2005 monitoring data, there are 85 wolves distributed throughout Estonia. Hunting is currently limited by quota; 37 animals were shot in 2004.

Data on wolf helminths in Northern and Eastern Europe are relatively scarce. The helminth fauna of wolves has been described for the European part of Russia (Jushkov, 1995), Lithuania (Kazlauskas and Prusaite, 1976), Poland (Soltys, 1964), and Belorussia (Shimalov and Shimalov, 2000). Distribution of the tapeworm *Echinococcus granulosus* (Hirvelä-Koski et al., 2003) and nematodes of the genus *Trichinella* among wolves has been studied in Finland (Oivanen et al., 2002) and Estonia (Järvis et al., 2001; Miller, 2003). In addition, the infection of wolves with *E. granulosus* has been reported in adjacent countries: Russia (Jushkov, 1995) and Lithuania (Kazlauskas and Prusaite, 1976). However, the sylvatic cycle of *E. granulosus* has been described only in Finland, identifying the wolf as a definitive host and the reindeer (*Rangifer tarandus*) and moose (*Alces alces*) as an intermediate host (Hirvelä-Koski et al., 2003).

Tapeworms of the genus *Echinococcus* are important parasites of mammals and the genus includes four species: *E. granulosus*, *E. multilocularis*, *E. oligarthrus*, and *E. vogeli*. *Echinococcus granulosus*, which causes the life-threatening disease cystic echinococcosis, is of significant medical and public health concern. The wolf is considered the principal definitive host for *E. granulosus* among wild carnivorous animals in Northern latitudes of Eurasia; moose and reindeer serve as intermediate hosts. The general domestic life cycle of *E. granulosus* involves dogs as the definitive hosts and livestock as intermediate hosts (Eckert et al., 2001).

Echinococcus granulosus is characterized as the most diverse species in the genus. Ten different genotypes (G1–G10) have been identified and categorized according to host and geographic range (McManus, 2002; Lavikainen et al., 2003). At least seven strains have been found in humans (G1, G2, G5, G6, G7, G8, and G9) (Thompson and McManus, 2002). As genetic diversity of *E. granulosus* seems to reflect differences in infectivity for distinct hosts (McManus, 2002), and as several strains are dangerous to humans, it is of great importance to use genetic methods for correct species and strain identification. These findings may have considerable implications for public health.

The aim of the present study was to provide the first data on helminth fauna of wolves in Estonia and to characterize the *E. granulosus* specimens obtained from them.

MATERIALS AND METHODS

Twenty-six wolf carcasses were collected from nine of 15 Estonian counties during the hunting seasons in 2000/2001 and 2003/2004. Carcasses were sexed and aged into two classes (young [<2 yr old, body weight <40 kg] and adult [>2 yr old, weight, >40 kg]) according to Aul et al. (1957).

Material was kept frozen until examination. Fecal samples were examined for helminth eggs by the flotation method. Muscle samples were taken from tibialis anterior muscle and examined by the compression method for *Trichinella* larvae. The identification of *Trichinella* species was carried out in the Trichinella Reference Centre. The random amplified polymorphic DNA analysis (RAPD) was used following the protocol of Bandi et al. (1995). Trachea, lungs, heart, intestinal tract, liver, gall bladder, kidneys, and urinary bladder were separated and examined according to recognized helminthological methods (Howie, 2000).

Helminths were fixed in Barbagallo's solution (Parre, 1985). *Echinococcus* specimens were preserved in 90% ethanol. All helminths were counted and identified. The identification of trematodes, nematodes, and cestodes was made according to Kozlov (1977). Cestodes of the genus *Taenia* and specimens of *E. granulosus* were determined according to Verster (1969) and Abuladze (1964), respectively. As no findings of mature *E. granulosus* have been documented in Estonia before, molecular genetic methods were used to confirm morphological identification. Specimens of parasites were deposited in the Zoological Museum of Tartu University, Tartu, Estonia, as Accession No. 1.

Two mature specimens of *E. granulosus*, obtained from a single wolf, were subjected to DNA extraction, PCR (polymerase chain reaction), PCR-RFLP (restriction fragment length polymorphism), and sequencing analysis. One mature *E. multilocularis*, obtained from the red fox (*Vulpes vulpes*) (Moks et al., 2005), was analyzed for comparison.

Total genomic DNA was extracted from *E. granulosus* with the use of a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) following manufacturer's protocol. Loci Eg9 and Eg16 were analyzed according to Gonzalez et al. (2002). The Eg9 locus was PCR amplified by using oligonucleotide primers PEg9F1 and PEg9R1, and the Eg16 locus with primers PEg16F1 and PEg16R1. PCR for Eg9 and Eg16 was performed in a total volume of 20 µl containing 20–80 ng of purified genomic DNA, 4 pmol of primers, 1X BD Advantage 2 PCR buffer (BD Biosciences, San Jose, California, USA), 1 U of BD Advantage 2 Polymerase Mix, 0.2 mM dNTP (Fermentas, Vilnius, Lithuania), and 1.5 mM $MgCl_2$. PCR cycling parameters were: 1-min denaturing step at 95 C, followed by 37 cycles of 30 sec at 95 C, 30 sec at 60 C, 40 sec at 68 C, and at the end the final extension 68 C for 7 min. Then 7 µl of the Eg9-PCR product was restricted for 5 hr with 10 U of either Cfo I (Roche Diagnostics) or Rsa I (New England Biolabs, Beverly, Massachussettes, USA) in a total volume of 10 µl according to manufacturer's protocol. The PCR and PCR-RFLP products were resolved by 2% agarose gel electrophoresis, visualized by ethidium bromide staining under UV light, and photographed.

A 529 bp fragment of the mitochondrial DNA NADH dehydrogenase 1 (mtND1) gene was amplified with primers NDfor1 5′AGATTCGTAAGGGGCCTAATA 3′ and NDrev1 5′ACCACTAACTAATTCACTTTC 3′ (Turcekova et al., 2003). Then 20–80 ng of purified genomic DNA and 4 pmol of primers were used for PCR. The PCR was performed in a total volume of 20 µl. Cycling parameters were 1-min denaturing step at 95 C, followed by 40 cycles of 30 sec at 95 C, 30 sec at 50 C, and 45 sec at 68 C, and at the end, 68 C for 7 min. Reactions were carried out in 1X BD Advantage 2 PCR buffer with 1U BD Advan-

TABLE 1. Prevalence and intensity of infection of helminths in Estonia wolves ($n=26$).

Helminth species	Number of animals infected	Minimum/maximum per animal
Trematodes		
Alaria alata	23 (89%; 76–99)[a]	3–1,533
Cestodes		
Diphyllobothrium latum	3 (12%; 2–26)	1–4
Mesocestoides lineatus	3 (12%; 2–26)	3–28
Taenia hydatigena	3 (12%; 2–26)	1–5
Taenia multiceps	7 (27%; 12–43)	2–68
Taenia ovis	4 (15%; 4–31)	3–12
Taenia pisiformis	2 (8%; 1–31)	2–5
Taenia spp.	5 (19%; 7–6)	1–3
Echinococcus granulosus	1 (4%; 0–14)	41
Nematodes		
Uncinaria stenocephala	20 (77%; 59–91)	1–46
Toxascaris leonina	2 (8%; 1–32)	1–11
Toxocara canis	2 (8%; 1–32)	1–2
Capillaria spp. (eggs in feces)	3 (12%; 2–27)	NC[b]
Trichinella spp. (larvae in muscle)	13 (50%; 32–69)	NC

[a] Prevalence and 95% confidence limits in parentheses.

[b] NC = not counted.

tage 2 Polymerase Mix, 0.2 mM dNTP, and 1.5 mM $MgCl_2$. The PCR product was purified with shrimp alkaline phosphatase and exonuclease I treatment. One unit of each enzyme (USB, Cleveland, Ohio, USA) were added to 10 µl of PCR reaction and incubated 30 min at 37 C, followed by a 15-min inactivation phase at 80 C. DNA cycle sequencing was performed with DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Uppsala, Sweden). Thirty-three cycles (15 sec at 95 C, 15 sec at 50 C, and 60 sec at 60 C) were performed in a volume of 10 µl. Sequences were resolved on an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, California, USA). The mtND1 fragment was sequenced in both directions with 5 pmol of primers NDfor1 and NDrev1.

Consensus sequences were created with the program CONSED (Gordon et al., 1998), with sequence data of both DNA strands. Sequences were double-checked by eye and aligned with Clustal W (Thompson et al., 1994). Program BioEdit was used as a sequence editor (Hill, 1999). In addition to the two *E. granulosus* sequences obtained in this study, eight *E. multilocularis* (seven from GenBank and one from Moks et al., 2005), 24 *E. granulosus* mtND1 sequences and outgroup sequences of *Taenia solium*, *E. vogeli*, and *E. oligarthrus* were included from the GenBank. MODELTEST version 3.06 (Posada and Crandall, 1998) was used to establish the model of DNA substitution that best fitted the data. Phylogenetic analyses were conducted with the use of a maximum-likelihood (ML) algorithm. ML analysis was performed with PAUP 4.0g10 (Swofford, 1998). Robustness was assessed by 1,000 bootstrap replicates.

The binary coefficient of Sorensen (Krebs, 1999) was used to compare the similarity in helminth fauna of local wolves and Eurasian lynx (*Lynx lynx*). Lynx data were obtained from a previous study (Valdmann et al., 2004b).

RESULTS

All animals were infected, and 13 species of helminths (one trematode, seven cestode, and five nematode species) were identified (Table 1). The most prevalent species were *Alaria alata* and *Uncinaria stenocephala*, which also showed the highest mean intensities, 303 and 11.6, respectively. The average number of gastrointestinal helminths per host was 325, ranging from 2 to 1,571. Both *Trichinella nativa* and *T. britovi* larvae were detected from muscle samples. Eggs of *Capillaria* spp. were found in three fecal samples, although no *Capillaria*

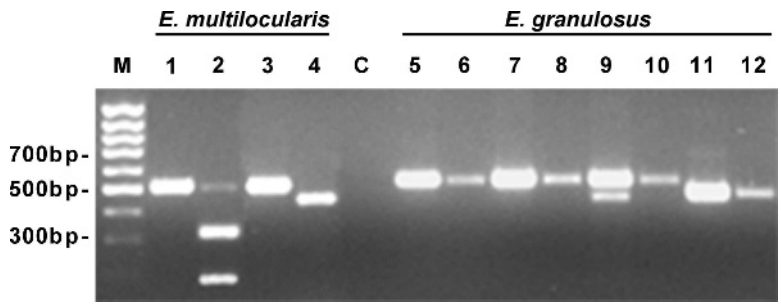

FIGURE 1. Diagnostic PCR-RFLP analysis for *Echinococcus granulosus* (lanes 5–12, two specimens in parallel). *E. multilocularis* (lanes 1–4, one specimen) is a control. Lanes: (M) Gene Ruler 100 bp DNA ladder; (1) *E. multilocularis* (E.mu)-Eg9 PCR; (2) E.mu-Eg9 + CfoI; (3) E.mu-Eg9 + RsaI; (4) E.mu-Eg16; (C) PCR negative control; (5 and 6) *E. granulosus* (E.gr)-Eg9 PCR, specimens 1 and 2; (7, 8) E.gr-Eg9 + CfoI, specimens 1 and 2; (9, 10) E.gr-Eg9 + RsaI, specimens 1 and 2; (11, 12) E.gr-Eg16 PCR, specimens 1 and 2.

worms were found at necropsy. Eggs of *U. stenocephala* and *Toxocara canis* were present in fecal samples. No differences in the intensity of infection were found among age and sex groups.

Echinococcus granulosus was identified in one wolf with the use of morphologic criteria as described by Abuladze (1964), and the identification was confirmed by genetic analysis with the use of total genomic DNA obtained from two specimens. Fragments of the Eg9 and Eg16 loci were successfully PCR-amplified, and sizes of the PCR products were similar to those obtained by Gonzalez et al. (2002) from *E. granulosus* (Fig. 1). When the Eg9 PCR product was cleaved with restriction enzymes (Fig. 1), Cfo I did not cut the Eg9 sequence for *E. granulosus*, but the Eg9 sequence for *E. multilocularis* was cut and gave exactly the same restriction pattern as described in Gonzalez et al. (2002). Restriction enzyme Rsa I had no recognition sequence within Eg9 locus for *E. granulosus* specimen 1, but for specimen 2, Rsa I gave a fragment that was slightly over 400 bp, demonstrating that the Eg9 sequence was somewhat different between the two *E. granulosus* specimens. The restriction enzyme Rsa I did not cut the *E. multilocularis* Eg9 sequence. Thus, PCR and PCR-RFLP confirmed that *E. granulosus* specimens were correctly identified using morphologic characters.

Both *E. granulosus* specimens gave identical sequences of 426 bp length when a fragment of the mtND1 gene was PCR-amplified and sequenced (sequence submitted to GenBank [AY842287]). When aligned with sequences from the GenBank, the *E. granulosus* sequence from Estonia was found to be identical with AF525297 (genotype G10), a mtND1 sequence of *E. granulosus* obtained from reindeer and moose in Finland (Lavikainen et al., 2003). MODELTEST selected the TrN+I+G model of DNA substitutions that best fitted the data. On a ML phylogenetic tree, *E. granulosus* mtND1 sequence from Estonia clustered with other *E. granulosus* sequences and was distinguished clearly from sequences of all other species, including *E. multilocularis* (data not shown). Topology of the phylogenetic tree was essentially the same as described by Lavikainen et al. (2003). The most remarkable feature of the phylogram is that geographically distant genotypes G10 (from Finland and Estonia) and G8 (from the USA) group together; their closest neighbors are genotypes G6 and G7. Genotype G10 differs from G8 and G6 by 12 mutations and from G7 by 14 mutations. The more distant genotype G5 is separated from G10 by 28 mutations.

Sorensen's binary coefficient of helminth fauna similarity between local wolves and lynx was 0.42.

DISCUSSION

All helminth species of wolves found in Estonia during this study have been found in adjacent countries—Russia, Lithuania, and Poland (Soltys, 1964; Kazlauskas and Prusaite, 1976; Kozlov, 1977; Jushkov, 1995). The number and species composition of helminths of the wolf in Estonia and neighboring areas is comparable as well. Similarity in helminth fauna is due most likely to similarity in diet. If available, wolves tend to feed on medium size ungulates (Okarma, 1995), but they also feed on smaller prey, especially in summer. Hares, small rodents, raccoon dogs (*Nyctereutes procyonoides*), and beavers (*Castor fiber*) form a significant proportion of the wolf's summer diet in Estonia (Valdmann et al., 1998).

The PCR-RFLP analysis of two specimens of *E. granulosus* (obtained from a single wolf) revealed different genotypes at the Eg9 locus (Fig. 1, lanes 10 and 11), implying that the wolf most likely acquired infection of *E. granulosus* from more than one source. The strain of *E. granulosus* from the wolf in Estonia carries an identical mtND1 sequence with the strain G10 identified from cervids (moose and reindeer) in Finland (Lavikainen et al., 2003). In addition to G10, another cervid strain, G8 from the United States (Minnesota and Alaska), has been obtained from moose, but also from humans (Bowles and McManus, 1993; McManus et al., 2002). Interestingly, *E. granulosus* genotype G8 from the USA (Minnesota) was the closest taxon to the genotype G10 from Finland and Estonia; and these haplotypes, together with genotypes G6 and G7, form a cluster on a phylogenetic tree (Lavikainen et al., 2003). Specificity of G8 and G10 to cervids is a likely explanation of their proximity on the phylogenetic tree. Because of the close phylogenetic position of mtDNA genotypes G6, G7, G8, and G10 and the fact that G6, G7, and G8 have been found also in humans, the G10 strain likely is a potential threat to the human population.

Two subspecies of *E. granulosus*, described by Sweatman and Williams (1963), occur in North America: *E. granulosus borealis* (North American origin) and *E. granulosus canadensis* (Scandinavian origin). Cervid genotype G10 is supposed to be related to *E. g. canadensis* (Lavikainen et al., 2003). Surprisingly, morphologic features (the number of segments and the length of the gravid proglottid) of *E. granulosus* G10 in Estonia were more similar to *E. g. borealis*. However, the incomplete set of morphologic characters (the wolf infected with *E. granulosus* was in poor post-mortem condition and all *E. granulosus* specimens were without hooks) and limited number of specimens of *E. granulosus* did not allow closer examination of subspecies status or analysis of the correlation between morphology and the mtDNA genotype. As there is large variation in morphological characters within the *E. granulosus* (Thompson and McManus, 2001), subspecies identification and correlation of phenotypes with genotypes requires an analysis of large number of specimens sampled all over the world.

The value of the calculated binary similarity coefficient of Sorensen (0.42) for wolf and lynx presumably reflects the presence of common prey species. Indeed, a high overlap of local wolf and lynx winter diet food niches (Pianka's coefficient was 0.9, Valdmann et al., 2005) most certainly provides a good ground for wolves and lynx to have similar helminth faunas. On the other hand, one would expect to have higher overlap in helminths, as the food niche overlap is remarkable. Relatively modest overlap in helmith faunas can be explained by different susceptibility of hosts to certain helminth species; different summer diets

of these predators may also impact their helminth faunas.

Populations of *E. granulosus*-infected wildlife can act as an important reservoir in promoting the transmission of the parasite to both domestic animals and humans. The general domestic life cycle of *E. granulosus* involves the dog as the definitive host and livestock as intermediate hosts (Eckert et al., 2002). Among domestic animals in Estonia, *E. granulosus* larvae were found only from pigs (Lešins, 1955). No infected dogs have been reported (Talvik, 1998; Jõgisalu, 2003). Considering the fact that cysts of *E. granulosus* have been found in moose in Estonia (I. Jõgisalu and T. Järvis, unpubl. data), it is conceivable that the wolf obtained the *E. granulosus* by consuming moose. Therefore, further studies are required to evaluate different cycles responsible for spreading echinococcosis in Estonia.

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