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***Echinococcus multilocularis* is a frequent parasite of red foxes (*Vulpes vulpes*) in Latvia**

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Summary

45 red foxes (*Vulpes vulpes*) from various regions of Latvia were examined by necropsy between 2003 and 2008 for the presence and infection intensity of *Echinococcus multilocularis*. The overall prevalence was 35.6 %, with the intensity of infection ranging from 1 to 1438. Although a limited number of foxes were examined, the prevalence was high and infected animals were found throughout the country. Considering the increasing number of humans infected with echinococcosis in Latvia since 2002, the situation is highly suggestive of a recent emergence of *E. multilocularis*. DNA sequences of four fox isolates were examined by multilocus analysis of mitochondrial and nuclear genes (*cox1*, *nad1*, *rrnS*, *atp6*, *actII*). All isolates were allocated to the predominant genetic form in Europe, with partial affinity of one isolate to a genotype that had previously been reported from southern Germany.

Keywords: *Echinococcus multilocularis*; Latvia; red foxes; prevalence; genotype

Introduction

Echinococcus multilocularis is the causative agent of alveolar echinococcosis (AE), which is rated as the most dangerous autochthonous parasitic zoonosis in Europe (Pawlowski et al., 2001). This potentially lethal disease, confined to temperate and cold areas of the northern hemisphere, appears to be emerging in European countries in the wake of increasing fox populations (Schweiger et al., 2008). The natural cycle of the parasite in central Europe is predominantly sylvatic, involving the red fox (*Vulpes vulpes*) as the main definitive host and a large number of species of arvicoline rodents as intermediate hosts (Eckert et al., 2001). Until the beginning of 1990's, the recognized geographical range of the parasite was confined to parts of Germany, Austria, Switzerland and France (Lucius & Bilger, 1995). Ecological changes during the past two

decades, coupled with successful vaccination of foxes against rabies, have resulted in a significant increase of the fox population and might have accounted for substantial extension of the parasite range (Romig et al., 2006). Meanwhile, reports of first findings and raised prevalence rates have been reported in a number of European countries (Duscher et al., 2006; Romig et al., 2006; Berke et al., 2008; Borecka et al., 2008). Important factors enhancing the risk of exposure to humans include increasing parasite prevalence and increasing number of infective eggs shed in the environment by definitive hosts (Kinčeková et al., 2006). A tenfold increase of parasite density (biomass) at the end of the 20th century has been estimated e.g. in south-western Germany (Romig et al., 1999).

Relatively little information is available about the pattern of environmental contamination from many recently recognized areas of *E. multilocularis* occurrence (hitherto regarded as non-endemic areas) in Europe including Latvia. In the context of the apparent emergence of the parasite in this country, the current study has focused on the survey of distribution and prevalence of the tapeworm in the main definitive host (red fox), and on genetic characterization of selected Latvian specimens in relation to the *E. multilocularis* genotypes circulating in Europe.

Materials and methods

Fox carcasses were obtained from hunters throughout Latvia (Fig. 1). A total of 45 red foxes (31 male, 14 female) were examined. They were classified as adults (35 animals) or juveniles (10 animals) according to the hunters' estimates. Animal carcasses were kept frozen until examination. The small intestine was separated and examined according to conventional helminthological methods. All helminths were removed and preserved in 90 % ethanol. *E. multilocularis* adult tapeworms were counted and identified based on the morphological and morphometric para-

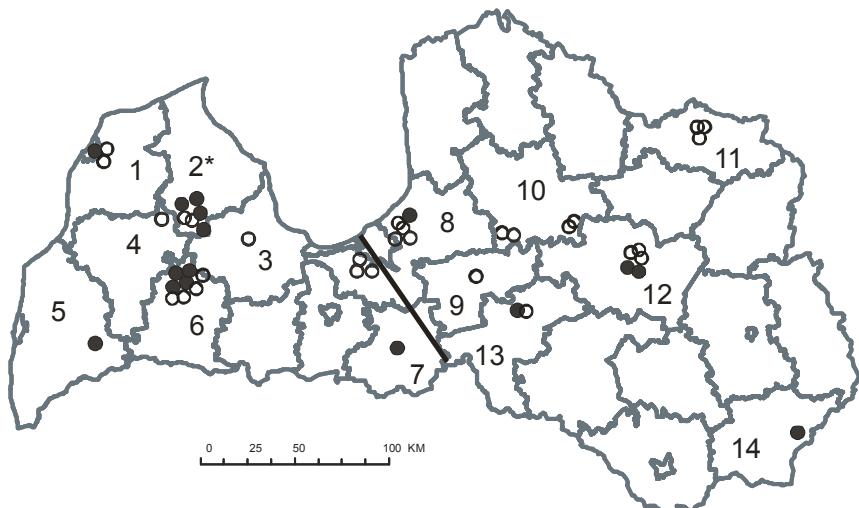


Fig. 1. Fox collecting sites (districts) in Latvia: 1, Ventspils ($n = 3/1$ infected animal with *E. multilocularis*); 2, Talsi ($n = 6/4$); 3, Tukums ($n = 1/0$); 4, Kuldīga ($n = 1/0$); 5, Liepāja ($n = 1/1$); 6, Saldus ($n = 8/4$); 7, Bauska ($n = 1/1$); 8, Riga ($n = 8/1$); 9, Ogre ($n = 1/0$); 10, Cēsis ($n = 4/0$); 11, Alūksne ($n = 3/0$); 12, Madona ($n = 5/2$); 13, Aizkraukle ($n = 2/1$); 14, Krāslava ($n = 1/1$).
 ○ - uninfected animal; ● - animal infected with *E. multilocularis*; * - origin of *E. multilocularis* isolates subjected to genetic analyses ($n = 4$);
 bold line - putative borderline between western and eastern part of Latvia

meters according to Kozlov (1977) and Abuladze (1964). Prevalence and infection intensity [mean intensity \pm standard error of the mean (S.E.M.)] were analyzed according to age, sex and location (western/eastern part of the territory of Latvia) using the Mann-Whitney test. SPSS for Windows was used for data processing and statistical analyses.

Genomic DNA was extracted from *E. multilocularis* parasites according to Šnábel *et al.* (2006), by using DNeasy tissue kit (Qiagen, Hilden, Germany). From Latvia, adults derived from 4 foxes in Talsi district were selected for genetic analyses. Five gene fragments (four mitochondrial and one nuclear) were amplified from DNA templates. The following primer pairs were designed to amplify mitochondrial portions of cytochrome *c* oxidase subunit (*cox1* target, 789 bp), NADH dehydrogenase subunit 1 (*nad1* target, 589 bp), and small subunit rRNA gene (*rnrS*, 362 bp): Em-COI-F: 5'-CCTGTTTGGCTGCTAT-3'; Em-COI-R: 5'-CCAGTAATCAACGGTCACCA-3'; Em-ND1-F: 5'-TGGTGGTTTGTTGGTTGGTT-3'; Em-ND1-R: 5'-TCCCTTCAGACTCCCCATA-3'; Em-12S-F: 5'-TGGTA CAGGATTAGATAACCCATT-3'; Em-12S-R: 5'-TTTAC CTTGTTACGACTTACCTCAG-3'. Parts of the mitochondrial ATPase subunit 6 (*atp6* target, 516 bp) and intron extract from the nuclear gene *EgActII* (*actII* target, 459 bp) were amplified by use of published *atp6* primers (Xiao *et al.*, 2005) and *ActK1/2* primers (Bart *et al.*, 2004). PCR products were purified using a Nucleospin Extract II kit (Macherey Nagel, Düren, Germany) and nucleotide sequences were obtained and analyzed using an ABI PRISM 377 automated sequencer (Applied Biosystems, USA).

Results and Discussion

Foxes infected with *E. multilocularis* were found in 9 of 14

sampled districts (Fig. 1). Among 45 examined foxes, 16 (35.6 %) were infected. The mean infection intensity (number of worms) \pm S.E.M. was 85.98 ± 37.8 , intensity varied from 1 to 1438 parasites per host animal. A higher number of infected animals was recorded from western part (24 examined/11 infected foxes) than from eastern part (21 examined/5 infected) of Latvia, but the difference was not statistically significant ($p = 0.128$) due to the small sample size. The intensity of infection related to geographical distribution was not also statistically significant ($p = 0.079$).

Latvian foxes had also recently been found infected with *E. granulosus* (Keidāns *et al.*, 2005). Such had only been reported before from two other European regions: southern England (Richards *et al.*, 1995) and the Iberian Peninsula (Segovia *et al.*, 2004).

In a survey conducted by the National Diagnostic Centre of Latvia, 12 of 57 raccoon dogs harboured *E. multilocularis* (Kirjušina, unpublished data). This confirms the potential importance of this host, which had already been found infected in other countries, e.g. Poland: among 78 raccoon dogs (*Nyctereutes procyonoides*) examined in northern Poland, 4 (5.1 %) were found to be infected (Gawor & Malczewski, 2005).

This neozootic, highly susceptible final host of *E. multilocularis* originated from eastern Asia and was introduced into the former Soviet Union, mainly the European part, in 1929 – 55 because it was considered a valuable fur animal (Lavrov, 1971). Altogether 9100 animals were released and the species is now very common in Finland, the Baltic states and many parts of eastern Europe, and can also be found in Germany and Sweden. It is still in a process of colonising the European territory, where it may be an increasingly important vector of *E. multilocularis* (Romig *et al.*, 2006; Sutor, 2008). In eastern Germany, the density of

the raccoon dog population will possibly in close future reach the high population density of foxes (Tackmann *et al.*, 2003).

Among neighboring countries of Latvia, *E. multilocularis* infection was recently documented in Lithuania, Estonia and Belarus. In Lithuania, the parasite was detected in 118 of 206 foxes (57 %), two of 34 domestic dogs (Bružin-

group deposited under GenBank reference AB018440) were detected, being manifested by differences in 3 bp in *cox1* and *atp6*, in 2 bp in *nad1*, and in 1 bp in *rrnS*. At the same time, a genetic cohesiveness between the European form of *E. multilocularis* and that derived from central China (Ningxia Hui Autonomous region, Gansu province) was recorded owing to shared species-variable nucleotides

Tab. 1 Nucleotide substitutions detected in *E. multilocularis* from Latvia relative to major European genotype

Isolate	<i>cox1</i> (789 bp)	<i>atp6</i> (516 bp)	<i>nad1</i> (589 bp)	<i>rrnS</i> (362 bp)	<i>actII</i> (459 bp)
L1	44C/T (ns), 728C/T (s)	205T/A (ns)	invariable	invariable	invariable
L2	invariable	invariable	invariable	invariable	invariable
L3	393T/G (s), 399G/T (s)	-	-	invariable	-
L4	-	invariable	invariable	-	-

"ns" - non-synonymous substitution; "s" - synonymous substitution; "-" not examined at given locus

skaitė *et al.*, 2007), and one of five muskrats (Mažeika *et al.*, 2003). Five of 17 foxes were recently found infected in Estonia (Moks *et al.*, 2005), as well as seven of 94 foxes in Belarus (Shimalov & Shimalov, 2002).

Human cases acquiring echinococcosis in Latvia have been registered since 2000. A total of 58 patients were diagnosed from 2000 to 2007, with rising numbers particularly since 2002 (6 – 15 patients per year) and a tendency to spread from the western and central to the eastern regions of the country (Keiss *et al.*, 2007). A similar trend in the number of human infections can be seen in neighboring Lithuania, where most cases of AE were documented in the past 5 years (10 – 16 cases/year in 2002 – 2006 compared with 0 – 4 cases/year in 1997 – 2001) (Bružinskaitė *et al.*, 2007). *E. multilocularis* thus appears to be of increasing human concern in Baltic countries. To obtain a better knowledge of the spatio-temporal development of the *E. multilocularis* distribution, the pattern of infection in foxes and raccoon dogs will be further studied in Latvia, in connection with thorough studies of aetiology of human infections.

A high degree of genetic uniformity is characteristic for *E. multilocularis*. Homogeneity was interpreted as the relatively recent divergence in species, which largely retained the ancestral life cycle with a conserved host spectrum (Haag *et al.*, 1997; Bart *et al.*, 2006). A sample panel of 25 isolates originating from 8 countries affected by parasite (Germany, France, Austria, Hungary, Poland, Latvia, Slovakia, Switzerland) had previously been sequenced in 5 genes (*cox1*, *nad1*, *rrnS*, *atp6*, *actII*) (Šnábel *et al.*, 2008). Two Latvian fox isolates (L1, L2) were among the samples which were examined in all five genes. Based on the evidence from a total of 2715 nucleotide sequences resolved in these markers and comparisons with GenBank® data, two major variants were determined in the northern hemisphere. Fixed nucleotide differences in European isolates compared to the form perpetuating in Japan and North America (representative sequence from Japanese/American

at *atp6* and *nad1* loci (Chinese data reported by Yang *et al.*, 2005 and GBR AY389984, respectively). This suggests that the dominant European variant circulates in a large part of the Eurasian continent. Within the European *E. multilocularis* sample, one Austrian isolate and four German isolates originating from a single site in Baden-Württemberg presented additional specific nucleotide exchanges across several genes. Interestingly, among 5 detected substitutions in 3 genes exhibited by the German genotype, one unique non-synonymous substitution 44C/T (inducing substitution of leucine with methionine) in *cox1* was identical to the L1 isolate from Latvia (Table 1). As this difference leads to amino acid change, it might have arisen independently by natural selection, possibly contributing to parasite virulence. However, the most likely explanation is that the Latvian and German isolates share ancestral species polymorphism. Besides the above shared mutation, specific 728C/T synonymous exchange (*cox1* target) and non-synonymous exchange 205T/A (responsible for substitution of leucine with methionine) in *atp6* were recorded in the L1 isolate. The L2 isolate conformed to the widely distributed European variant in all examined sequences. Within two other Latvian isolates subjected to analyses in some of gene targets, two specific (synonymous) mutations 393T/G and 399G/T in *cox1* were found in the L3 isolate, whereas the L4 isolate produced the pattern entirely identical to the major European variant of *E. multilocularis*. Genetic diversity in parasites was higher in the historically documented central European endemic zone (0.082 % of polymorphic sites in examined gene fragments) than in Latvia (0.052% of polymorphic sites) which is presumed to be invaded with the tapeworm more recently. Coupled with the commonly distributed transversal profiles of sequencing patterns even in apparently newly endemic parts of Europe, our data support a view of Knapp *et al.* (2007) inferred from microsatellite patterns, who stated that *E. multilocularis* focus in Europe is governed by a “mainland-island” transmission model, where

ancestral foci supplied hitherto non-endemic areas by dispersal generated by fox mobility and migration.

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