Veterinary Parasitology 174 (2010) 241–246

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Spatial distribution and genetic diversity of *Echinococcus multilocularis* in Hungary

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ARTICLE INFO

Article history:
Received 17 March 2010
Received in revised form 5 August 2010
Accepted 19 August 2010

Keywords:
Echinococcus multilocularis
Red fox
Vulpes vulpes
Molecular epidemiology
Genetic polymorphism
Tandem repeated multi-locus microsatellite
Hungary

ABSTRACT

Human alveolar echinococcosis, caused by the tapeworm *Echinococcus multilocularis,* is the most pathogenic helminthic zoonosis in the temperate and arctic region of Europe. Between November 2008 and February 2009, 840 red fox (*Vulpes vulpes*) carcasses, were randomly collected from the whole Hungarian territory. The intestinal mucosa from all the foxes was tested by sedimentation and counting technique. *E. multilocularis* adult worms were detected in foxes of 16 out of the 19 Hungarian counties and in the suburban areas of the capital, Budapest. The prevalence and abundance of infection was significantly ($P < 0.001$) higher in the north-western half (16.2%, CI = 14.5–17.9; $m \pm SE = 165.5 \pm 112.4$) than in the south-eastern half of the country (4.2%, CI = 3.2–5.2; $m \pm SE = 3.6 \pm 2.1$). The highest prevalence (26.6%, CI = 22.5–30.8%) and abundance ($m \pm SE = 614.2 \pm 469.3$) was observed in the Northern Mountain Region bordering Slovakia. The multi-locus microsatellite analysis of 81 worms showed the presence of four out of the five main European profiles. The H profile was the most common profile (55.5%) with nine genotypes, followed by the G (18.5%) with two genotypes, E (13.6%) with one genotype and D (12.4%) with two genotypes. The genetic distance was not statistically correlated with the geographical distance of the samples, supporting the hypothesis that the geographical distance is only a minor factor among those involved in the genetic distribution of this parasite in Europe. These data indicate that Hungary should be considered as a peripheral area of a single European focus, where the dispersal movement of foxes resulted in the spreading of the parasite from one county to another within a time period short enough to avoid a substantial genetic drift.

1. Introduction

Human alveolar echinococcosis (AE), caused by the tapeworm *Echinococcus multilocularis*, is the most pathogenic zoonosis in the temperate and arctic region of Europe (Kern et al., 2003). Until the end of the 1980s, *E. multilocularis* endemic areas in Europe were known to exist only in eastern France, Switzerland, southern Germany, and western Austria, and the number of human cases was limited (Kern et al., 2003). In the last 20 years, the population size and the infection rate of the red foxes (*Vulpes vulpes*) increased; several new endemic foci were detected in these countries, resulting in an emerging epidemic of AE 10–15 years later the infection in the foxes (Schweiger et al., 2007). This parasite was also reported from 13 countries surrounding the historical endemic area, and the parasite is currently known to be endemic in 17 countries of the European Union (Romig, 2009; Rataj et al., 2010; Sikó Barabási et al., 2010). Although the number of human cases of AE might be still underreported, several autochthonous cases were described in humans in the newly endemic countries in recent years, and this number

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doi:10.1016/j.vetpar.2010.08.023

is likely to increase in the future (Romig, 2009). In Hungary, the photograph and the description of macroscopic lesions (two fist-sized, undulating cysts) in one of the presumed report of alveolar echinococcosis written by two surgeons (Jakab and Faller, 1988), clearly indicate that the case was indeed cystic echinococcosis. The only confirmed case of human AE reported from Hungary cannot be considered autochthonous as the patient visited Austria, a historical endemic area, several times in the past decades (Horváth et al., 2008). Although autochthonous AE has never been described in Hungary, the parasite was recently detected in red foxes in seven northern counties with a low overall prevalence (Sréter et al., 2003, 2005). These data suggest the spreading and emergence of *E. multilocularis* in Europe including Hungary. Nevertheless, it cannot be completely excluded that identified eastern and western endemic regions have long existed and were detected only recently due to increased disease awareness.

Molecular tools should contribute to the understanding of the epidemiology of this parasite in Europe. To date, various molecular markers have been applied to the study of the genetic polymorphism of *E. multilocularis*, revealing very little variation within this species (Bowles et al., 1995; Bretagne et al., 1996; Rinder et al., 1997; McManus, 2006). However, microsatellite analysis seems to be a good molecular tool for the detection of genetic polymorphisms within *E. multilocularis* populations (Nakao et al., 2003; Bart et al., 2006; Knapp et al., 2007, 2008, 2009; Casulli et al., 2009).

The aims of this study were to detect the prevalence and the distribution of *E. multilocularis* in Hungary, to identify genotypes circulating in the country, and to analyze these findings in light of recent European epidemiological and molecular genetic data.

2. Materials and methods

2.1. Sample collection and parasitological examination

The fox sample size (1% of the total fox population) used in this study was previously established on the basis of the National Game Management Database (www.vvt.gau.hu/adattar/). From November 2008 to February 2009, carcasses of red foxes sent to the Veterinary Diagnostic Directorate, Budapest, in connection with the rabies immunization and control program, were included in this study. The animals were individually labelled with an identification number reporting the information on the locality and date of collection. Carcasses were forwarded in individual plastic bags at +4 °C. Red fox carcasses ($n = 840$), representing more than 1% of the total fox population of each county, were randomly selected from 19 counties and from the Budapest municipality (covering 100% of the Hungarian territory, 93,029 km^2; Fig. 1). The intestinal tract was removed and stored at −20 °C. For safety reasons, the intestinal tract was frozen at −80 °C for 10 days before examination. After freezing, the gut was thawed at room temperature, the intestinal mucosa was collected and tested by sedimentation and counting technique (SCT) according to a previous published protocol (Deplazes and Eckert, 1996). Adult worms of *E. multilocularis* were identified by morphology as previously described (Sréter et al., 2003). The intensity of infection was determined by counting of worms in low level infections (<100 worms/fox) or by estimation of worm number by dilution and counting technique in high level infections (≥100 worms/fox). For molecular studies, 1–25 worms were collected from each infected foxes and stored in 70% (v/v) ethanol. The prevalence, the confidence interval, and the freedom from infection, based on the sample size, were calculated by Survey Toolbox 1.04 (Cameron, 1999; www.ausvet.com.au/). The Fisher's exact test and the Mann–Whitney test were performed by InStat 3.0 (GraphPad Inc., La Jolla, CA). Differences were considered significant when $P < 0.05$.

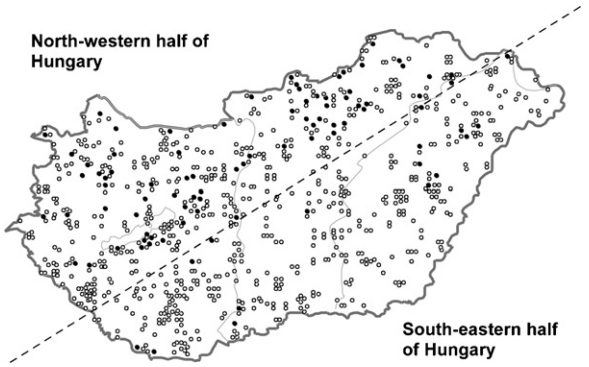

Fig. 1. Distribution of *Echinococcus multilocularis* infected red foxes (*Vulpes vulpes*) ($n = 90$; filled circles) and uninfected red foxes ($n = 750$; open circles) collected in Hungary between November 2008 and February 2009.

2.2. DNA extraction, fluorescent PCR amplification, and fragment analysis

For the molecular identification of the parasites, only one worm was analyzed from each positive fox. Each single worm was then washed extensively in sterile distilled water to eliminate host and/or faecal contaminants. The genomic DNA was extracted from single adult worms, purified and concentrated in 30 μl by Wizard Magnetic DNA Purification System for Food (Promega, Madison, WI), according to the manufacturer's instructions. After purification, DNA samples were stored at −20 °C. The genetic diversity of *E. multilocularis* was assessed by fluorescent PCR followed by fragment size analyses with the tandem repeated microsatellite target EmsB (GenBank accession no. AY680860). The PCR amplification was performed in a 30 μl reaction mixture including 4 pmol of the forward 5′-(6-FAM)-fluorescent labelled primer (Primm, Milan, Italy); (EmsB A 5′-GTGTGGATGAGTGTGCCATC-3′), 7 pmol of the unlabelled reverse primer (EmsB C 5′-CCACCTTCCCTACTGCAATC-3′), 2× Master Mix (Promega), and 2.5 μl of genomic DNA. The PCR amplification was performed in a GeneAmp 9700 thermocycler (Perkin Elmer, Norwalk, CT), under the following conditions: denaturation step at 94 °C for 10 min, followed by 45 cycles with denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, and a final elongation at 72 °C for 30 min. The reproducibility of the EmsB amplification was tested by performing each PCR reaction two times under identical conditions. The presence of PCR prod-