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Epidemiological coherency of vulpine dirofilariosis in environmental conditions of Slovakia

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Summary

Dirofilariosis belongs to zoonotic vector-borne diseases with fastest spread into new areas caused by extreme weather and seasonal changes in climate. In Slovakia, *Dirofilaria* spp. parasites affect more than 30 % of dogs living in endemic regions in southern parts of territory, however, data on wildlife circulation of this parasite are still scarce. In order to clarify the role of red fox (*Vulpes vulpes*) as the most abundant canid species in Europe in maintaining the parasite in natural foci, an initial survey of dirofilariosis in this carnivore species was conducted in Slovakia. The samples of 183 red foxes hunted in 2007 – 2009 in regions of South-Eastern and Northern Slovakia with different geographical and climate characteristics were examined by means of PCR method using specific *D. repens*, *D. immitis* and *Acantocheilonema recognitum* primers. The DNA was isolated from spleen samples using commercial kit and PCR approach was used for diagnostics. After amplification selected products were purified and sequenced to elucidate any homologies with previously deposited sequences in Gen Bank. The results showed 105 out of 183 examined specimens (57.4 %) being infected, with great regional differences in prevalence. Phylogenetic relationships within *Dirofilaria* species indicate that obtained isolates belong to *D. repens*. The results confirmed the role of red foxes as the reservoir of parasite. Herein, epidemiological factors that may be coherent with the *Dirofilaria* parasites distribution and circulation in wildlife and implications in risk assessment and prevention for domestic animals and human are discussed.

Keywords: Canidae; red fox; *Vulpes vulpes*; *Dirofilaria*; epidemiology

Introduction

The incidence and spread of vector-borne diseases is linked to extreme weather events and seasonal changes in micro- and macroclimate. According to Intergovernmental Panel on Climate Change (2007) the global surface temperature increased approximately by 0.3 – 0.6 °C during the 20th century with considerable effect on the hydrological cycle (heavy rainfalls, floods, mega storms, heat waves, droughts) and biosphere (deforestation, desertification, coral bleaching) as well as pole ward and upward shifts in ranges in plant and animal species. Variations in biodiversity together with anthropogenic effects on ecosystems may be accompanied by spread of tropical insects followed by pathogen transmission (Zell, 2004). As arthropod vectors, their reproduction and mortality are significantly responsible to seasonal climate changes, the dynamics and transmission patterns of vector-borne diseases is very likely to be influenced.

This scenario begins in recent years to take place in Europe, where mainly due to warming and frequent flooding accompanied by increased incidence of mosquitoes several vector-borne diseases began to spread, including filarioses. Disease agents in Europe are several filarial species (Filaroidea) that are infectious for carnivores, i.e. *Dirofilaria immitis*, *D. repens*, *Acantocheilonema recognitum*, *A. dracunculoides* and *Cercopithifilaria grassi* (Genchi *et al.*, 2011). Their transmission is associated with arthropods, which in the parasite life cycle play the role of both, vector and intermediate host. The most frequently occurring species within the European territory are *D. immitis* and *D. repens* transmitted by different types of mosquitoes (e.g. *Aedes* spp., *Anopheles* spp., *Culex* spp.). Definitive hosts constitute carnivores, with the greatest contribution of representatives of the family Canidae. Humans can become an occasional host; however the incidence of human infections in Europe has markedly accrued recently. *Dirofilaria immitis*, with a cosmopolitan distribu-

tion, and *D. repens*, only reported in the Old World, are the main causative agents of zoonotic filariosis. Adult parasite living in the organism of definitive host produces larval stages (microfilariae) that circulate in the blood. During blood sucking microfilariae get into the mosquitoes body where they develop to infectious L3 larvae and during repeated blood sucking the infective stages enter next host, where they shed and mature to adults. Adult worms from both species live in the pulmonary artery and in subcutaneous tissue of dogs and cats, resulting in the production of blood-circulating microfilariae in dogs, while amicrofilaremic infections are common in felines. In humans, worms cannot reach maturity, and preadult worms are responsible for pulmonary (*D. immitis*) and for subcutaneous or ocular (*D. repens*) dirofilariosis (Simon *et al.*, 2009).

Short period of development during seasons of high ambient temperatures allows releasing of several generations and increases potential parasite temporal availability which escalates the risk of infection. Therefore, this parasite formerly occurred endemically mainly in regions with tropical, subtropical and prevailing warm climate. As a result of climate change, global warming and frequent flooding followed by an increased incidence of mosquitoes extended the parasite range also to other areas of Europe (Morgan & Wall, 2009).

The incidence of dirofilariosis agents and the emergence of new endemic areas were recorded in recent decade in several European countries with mild climate, such as Bulgaria, Serbia, Hungary, Austria, Czech Republic, Poland, Russia, or Latvia (Simon *et al.*, 2009; Fók, 2012). The first occurrence of the disease in dogs in Slovakia was recorded in the western part of territory in 2005 (Svobodová *et al.*, 2005). The results of systematic survey performed in 2007 showed a high prevalence (34.5%) in dogs, originating from lowland areas of southwestern and south-eastern Slovakia, characterized by the warmest climate in our country (Miterpáková *et al.*, 2008).

Although in recent years intensive research on dirofilariosis in domestic carnivores (dogs, cats) has been in the progress, data on parasite distribution in populations of wild carnivores are rare. Red fox (*Vulpes vulpes*) is the most abundant species of wild canid predators in major part of Europe and often occurs in the vicinity of human settlements. Like other wild and domestic carnivores foxes may become infected with several species of filarial nematodes. Since in Slovakia *D. repens* and *D. immitis* infections in dogs were reported, we conducted an initial survey of dirofilariosis in red foxes in geographically and climatically different areas in order to clarify their role in maintaining the parasite in natural foci.

Materials and Methods

Studied areas

Foxes were hunted in 2007 – 2009 in regions of south-eastern and northern Slovakia with different geographical and climate characteristics (Fig. 1).

The major part of south-eastern Slovakia represents the Eastern Slovak Lowland, expanding on 2500 km² bordering with Ukraine and Hungary (49°11' – 48°24', 21°51' – 22°12' E). The altitude of the area is 95 - 200 m above sea level. Region belongs to the mild climatic zone and has generally a warm and fairly dry cli-

mate with an average annual air temperature of about 9 – 10 °C, with 50 – 70 summer days and warmest month being July with average temperature +19.7 °C. The annual rainfall achieves 600 up to 750 mm and profound rainstorms often occur, causing intensive floods. The area is characterized by fan shaped stream network with important rivers Bodrog and its tributaries Ondava, Laborec, Latorica, Uh and Topľa. A big water reservoir Zemplínska Šírava (33 km²) and system of protective dikes and drainage canals have been built to prevent frequent floods. Lowland meadows, grasslands, pastures and fields with swamps and swamp forests prevail in the landscape, with protected habitats in nature preserves. Aforementioned conditions are associated with recurring mosquito calamities.

Northern Slovakia is mountainous and coldest area of Slovakia (48°47' – 49°53', 18°48' – 22°51' E) with average elevations 300 – 800 m above sea level and mountain peaks reaching up to 2655 m a.s.l. (Gerlach). More than 50 % of the landscape comprise forest complex, land principally occupied by agriculture with significant areas of natural vegetation - natural grasslands and sparsely vegetated areas and alpine environment in the Tatra Mountains. Due to location and terrain with highly variable altitude the climate of the region is very diverse. Mountain ranges are significant climate ranges of the region and together with rugged terrain substantially affect particular components of climate. Studied location lies largely in the area with cold continental climate, the river (Orava, Váh, Poprad) valleys have a slightly warmer climate and belong to moderately warm climate zone. The average annual temperature is 2.4 – 6 °C and decreases with higher altitude. In elevation 1000 m a.s.l. reaches the average values of 4 to 5 °C, in 2000 m is about -1 °C and on the High Tatras peaks is in average below -3 °C. Average summer temperature in July and August in basins reaches 16 to 18 °C, in the mountains, depending on altitude, less than 15 °C. The number of summer days with temperatures above 25 °C is small: 20 to 40, and 0 in the hilltops. Mountains in northern Slovakia are generally richer in atmospheric precipitation (up to 2000 mm), relatively low rainfall is in localities in so called "rain shadow of the mountains" (less than 350 mm). The average rainfall in the area is 550 – 700 mm.

Sample collection

In total 183 red foxes (*Vulpes vulpes*) as the most abundant free-living carnivores were hunted during the monitoring of antirabic vaccination effect. 110 individuals were captured in southeastern Slovakia (Košice region), 73 specimen originated from northern part of territory (Žilina and Prešov regions). The hearths, lungs, and spleens were removed by the dissection at the State Veterinary and Food Institutes, delivered to the Institute of Parasitology SAS and stored by -20 °C until examination.

Methods used

During helminthological autopsy the hearts and lungs were examined for the presence of adult *D. immitis*. Due to lack of fresh blood and animal carcasses *Dirofilaria* spp. survey was based on molecular techniques. The total genomic DNA for PCR analyses was extracted from fox spleens using commercial isolation set DNeasy Blood and Tissue Kit (Qiagen®). The amplification of the fragments of cytochrome oxidase subunit 1 (CO1) gene was

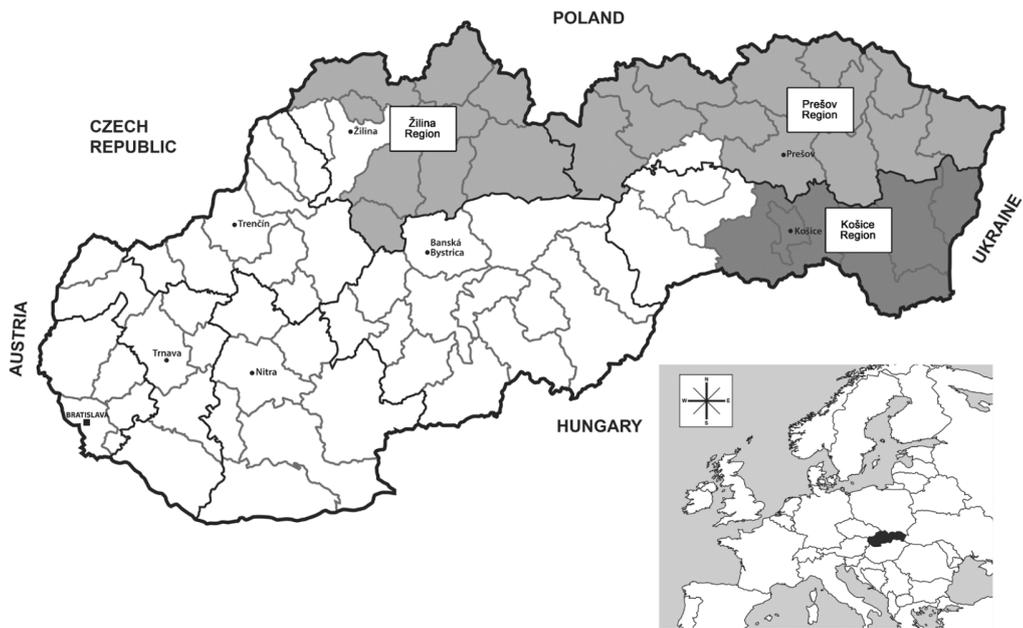


Fig. 1. The origin of foxes investigated for dirofilariosis in Slovakia

performed using specific *D. repens*, *D. immitis*, and *Acantocheilonema recognitum* pairs of primers according to Rishniw *et al.* (2006).

After amplification selected products (one isolate from three different localities, i.e. Košice, Michalovce and Prešov) were purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and sequenced on Applied Biosystems ABI PRISM 3100-Avant DNA Sequencer. In order to elucidate any homologies with previously deposited sequences in Gen Bank, we conducted a BLAST search (www.ncbi.nih.gov/BLAST/). Next the multiple alignment was done by the use of CLUSTAL W implemented to MEGA 5.0 package (Tamura *et al.*, 2007). The neighbor-joining (NJ) tree was constructed using MEGA 5.0 software. The evolutionary history of the isolates from Slovakia was inferred using the Maximum Likelihood method. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2007).

Results

No adult *D. immitis* worms were found during helminthological autopsy of 183 fox lungs and hearts.

The PCR analyses using species specific primer pairs revealed the presence of *D. repens* DNA in 105 out of 183 investigated foxes, *D. immitis* and *A. recognitum* were not confirmed in fox samples. The overall prevalence of vulpine dirofilariosis of 57.4 % with great regional difference ($\chi^2 = 53.163$; $p < 0.001$) was observed. Most positive foxes (87 out of 110) were hunted in region of the south-eastern Slovakia, where the prevalence reached 79.1 %. Also in northern, highland region 18 of 73 (24.6 %) of foxes were infected with *D. repens* (Fig. 1).

As a result of sequencing of partial gene of COI we obtained the sequences similar in length and amounting respectively: *D. repens* MI – 209 bp, *D. repens* KE – 203 bp and *D. repens* PO – 202 bp. The BLAST analysis of our three sequences show 100 %

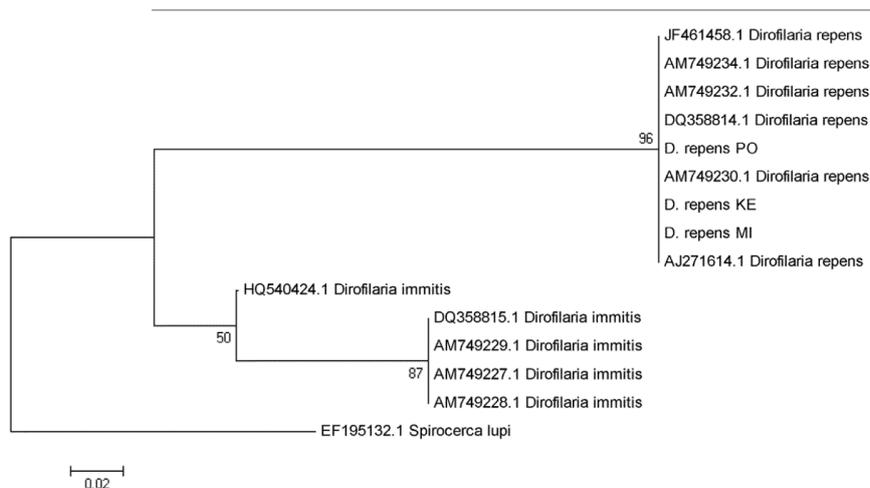


Fig. 2. Reconstruction of phylogenetic relationships within *Dirofilaria* based on partial sequences of COI mtDNA. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model, the bootstrap consensus tree inferred from 1000 replicates

similarity, in the overlapping regions, between all isolates from foxes and with sequences of *Dirofilaria repens* isolated from humans (AM749234, JF461458), cats (AM749232) and dog (AM749230). The evolutionary history based on COI was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Fig. 2). The final alignment of COI sequences of the representatives of *Dirofilaria* was 202 bp long, where 41 characters were variable and 24 parsimony informative. The out-group for phylogenetic analysis was *Spirocerca lupi* (EF195132.1) (Fig. 2).

Discussion

The results of epidemiological research on vulpine dirofilariosis showed 105 out of 183 examined individuals from Slovakia (57.4 %) being infected with *D. repens*, that confirms that red fox as the most abundant wild canine predator is an important natural reservoir of the parasite. Several authors affirm the role of this carnivore in epidemiological chain of dirofilariosis (Mañas *et al.*, 2005; Magi *et al.*, 2008, 2009; Marks & Bloomfield, 1998). Dirofilariosis caused by *D. immitis* was also detected in other wild carnivore species – in wolves, coyotes, dingoes, raccoons, and black-footed ferrets (Pascucci *et al.*, 2007; Custer & Pence, 1981; Starr & Mulley, 1988; Nakagaki *et al.*, 2000; Wisely *et al.*, 2008). A beech marten naturally infected was also found in Slovakia (Miterpáková *et al.*, 2013). These findings indicate that the role of natural reservoir may play many carnivore species that occur in endemic areas.

The red fox is one of most numerous free living carnivores in Europe. A significant increase of vulpine population was in last decades observed in almost the whole European area after the oral rabies vaccination campaigns. Similar situation was recorded also in Slovakia. While in 1993 the spring stock was 6 154 animals, in 1996 this number increased to 13 331 foxes, 18 920 in 2003 and 29 530 individuals were counted in spring stock in 2011 (National Forest Centrum, 2011). From the epidemiological point of view, multiple accumulation in fox numbers and their urbanization mean, that the host is most accessible both for vectors and parasites, which may result in the increase in prevalence. Our finding of the large numbers of foxes infected with *D. repens* adverts to the role of this host in the circulation and transmission of dirofilariosis in wildlife. Magi *et al.* (2008) reported that in central Italy is in foxes more incident genus *Acantocheilonema* as *Dirofilaria* species and assume that this is due to the distribution of ticks as vectors of filariae from the genus *Acanthocheilonema*, whereas mosquitoes that transmit *Dirofilaria* spp. are in this area regularly and targeted regulated. Nevertheless, this is not the case in Slovakia, where the mosquito population is not controlled. Most noticeable is the situation on the lowlands in the south of territory, where floods occur frequently accompanied by mass occurrence of mosquitoes.

According to monitoring of mosquito species composition, 23 out of 50 in Slovakia known species are ranging in the Eastern Slovak territory. The most important are blood sucking plague with calamity occurrence, i. e. *Aedes vexans*, *Culex pipiens*, *Ae. cinereus*, *Ae. rossicus*, *Ochlerotatus sticticus*, and *O. cantans* with the first two mentioned being the most frequent species also in other parts of Slovakia (Strelková & Halgoš, 2012). Further-

more, Bocková *et al.* (2013) using molecular approaches diagnosed the presence of *D. repens* DNA in pooled samples from *Ae. vexans* from eastern Slovakia. *Ae. vexans* is an inland floodwater mosquito species that habits in yards, fields, wooded areas and bites during night, at dusk and dawn but in shaded areas also during day. It has been proved that *Ae. vexans* bloodmeals most frequently from mammalian hosts, including canids and humans thus this species is a suitable vector for *Dirofilaria* transmission. Mosquitoes from the genera *Aedes* and *Culex* may produce multiple generations, whereas *Aedes vexans* utilizes fresh floodwater habitats and lays desiccation-resistant eggs in ground depressions inundated by fresh floodwater. According to Trpiš and Shemanchuk (1970), the most suitable temperature for larval development of *Aedes vexans* is 25 °C. At this temperature, 86 % of the larvae survive and pupate in 7 days. At 10°, 15°, 20°, and 30 °C larval development time is 46, 22, 10, and 8 days. In water at 25 °C the adults emerge in 2 to 5 days. It stands to reason that long periods of high temperatures enable the development of several generations of the vector during the summer season, when conditions are also suitable for the rapid development of parasite larval stages, leading to increased parasite biomass in the environment and escalating the likelihood for formation of new natural foci.

The development of larvae in intermediate host depends significantly upon environmental temperature, which must be higher than 14 °C. At this temperature, larvae in the mosquito's body merely survive without developing further. With increasing temperature increases also the rate of development. At 16 °C infectious stages appear after 43 days, at 22 °C in 16-22 days, and at temperatures above 28 °C as early as after 3-4 days (Fortin & Scolombe, 1981).

The result of our initial survey of dirofilariosis in red foxes showed a high prevalence of the disease in the carnivore population from southeastern Slovakia (79.1 %), in the area, where climatic conditions favor both, the occurrence of vector and parasite development in its organism (Iglódyová *et al.*, 2012). Previous research confirmed in this area 34.5 % of dogs being infected with subcutaneous dirofilariosis and the presence of *D. immitis* was also confirmed. Finding of 24.6 % infected foxes in northern and colder regions, where already also autochthonous cases of canine dirofilariosis were recorded adverts to the substantiality that the environmental circumstances in these areas are also favorable for the presence of both, parasite and vector.

According to data from the Slovak Hydrometeorological Institute, during the study period 2007-2009 above-normal summer temperatures were observed. Actually, the average daily temperature in July and August 2007 and 2008 overcame historical maximum since 1871 and the deviation from the long-term average reached +2 to +3.1 °C throughout entire Slovakia. Furthermore, in these year was above-average warm June when also in northern regions temperatures in valleys reached +15 °C and higher. During summer seasons occurred several periods of a sequence of very warm even tropical days with temperatures over 25 °C and frequent storm after heat spells, which allowed the development of infective larvae and over-infestation of vectors. Here at, in mountains with plenty of wooded areas the desiccation of temporary water reservoirs during prolonged dry with heat days is less intense than in agricultural areas with cultural steppe, which

favors the continuation of both, vector and parasite life cycles and extends the period of infection risk.

Another factor that has to be taken into consideration for a comprehensive assessment of dirofilariosis epidemiology is the possibility of parasite importation via infected dogs. In the northern part of Slovakia there are several natural parks that are favorite destinations for tourist from our and neighboring countries. People are during outdoor activities often accompanied by their dogs and it is possible that *D. repens* was introduced into northern region by infected dogs from endemic regions. Abundance of vectors, wildlife reservoir hosts and suitable environmental conditions allowed the continuation of parasite life cycle in natural conditions, practically beyond control.

All above mentioned circumstances are inevitably to be considered for the proper timing of chemoprophylaxis in domestic carnivores to provide the protection against infection. In these new-formed endemic foci, in particular, it is important to create awareness of professional public as previous findings disclosed a very low level of information in this important group.

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