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ORIGINAL PAPER

Potential mosquito (Diptera:Culicidae) vector of *Dirofilaria repens* and *Dirofilaria immitis* in urban areas of Eastern Slovakia

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Abstract This paper follows the study from 2013 focused on the molecular screening of mosquitoes as vectors of *Dirofilaria* spp. which provided the information on *Aedes vexans* as a potential vector of *Dirofilaria repens* in Slovakia. Current entomological and molecular research indicates that *Ae. vexans* can participate also in the transmission of *Dirofilaria immitis* within the region. Using the standard PCR method, we examined 10,500 mosquitoes (*Ae. vexans*, *Ae. rossicus*, *Anopheles maculipennis* s.l., *Ochlerotatus sticticus*, *Ochlerotatus cantans*, *Ochlerotatus caspius*, *Culex pipiens/Culex torrentium*, *Coquillettidia richiardii*), collected using CO_2-baited traps at six locations in the Eastern Slovakia. Out of 105 pools, 6 pools of mosquitoes *Ae. vexans* were positive for *D. repens* DNA (minimum infective rate in *Ae. vexas* was 6:6.900, i.e. 0.8 per 1.000 mosquitoes), within which 4 were concurrently positive for *D. immitis* (minimum infective rate in *Ae. vexans* was 4:6.900 i.e. 0.5 per 1.000 mosquitoes).

Keywords *Aedes vexans* · Mosquito vectors · *Dirofilaria repens* · *Dirofilaria immitis* · Slovakia

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Introduction

Dirofilaria repens and *Dirofilaria immitis* belong, from the epizootological and zoonotic point of view, to the most important filarial species in Europe. Their spread from endemic southern European areas to the Eastern and Western Europe is facilitated mainly by global warming. Until now, their occurrence in areas further north has been sporadic and connected especially with imported cases (Klintebjerg et al. 2015; Saevik et al. 2014).

Transmission and geographical extent of a parasite is determined by the presence of a competent vector. A large part of the knowledge on vectors of *Dirofilaria* spp. is based on the experimental laboratory research (Latrofa et al. 2012), upon which it is assumed that more than 70 mosquito species are potentially competent to perform the transmission even in natural conditions (Cancrini et al. 1995; Vezzani and Carbajo 2006; Vezzani et al. 2011). Vectorial capacities of individual species, however, differ in individual geographical conditions, depending on various factors, and the transmission of *D. repens* and *D. immitis* in different areas can be of a seasonal nature. In comparison with areas with the tropical climate, in a temperate zone, it is often limited to the warmest months of the year, which usually corresponds to activity peaks and population maximums of (potential) vectors.

A prevailing filarial species in Europe (excluding the northern Italy) is *D. repens*. While in the countries of southern Europe the infections in dogs and humans have been observed for several decades, in the last decade, they occur in the central and western Europe in countries like Hungary, Poland, Austria, Czech Republic, and Slovakia (Fok 2007; Demiaszkiewicz et al. 2009; Masny et al. 2011, 2013; Cielecka et al. 2012; Duscher et al. 2009; Svobodová 2006;

Ondriska et al. 2010, 2014). Incidence of dirofilariosis caused by *D. immitis* is disproportionately lower; however, sporadic cases of autochthonous infections in dogs are observed (Jacsó 2009). Therefore, in these regions, the stronger emphasis is recently being put on examination of vectors using non-molecular (zootomy) and molecular methods to identify competent and potential vectors. DNA of *D. repens* was detected in mosquitoes *Aedes vexans* in Slovakia, in the Czech Republic, and Germany (Bocková et al. 2013a, c; Rudolf et al. 2014; Sassnau et al. 2014). Recently, the DNA of *D. immitis* was detected in Germany in mosquitoes *Culex pipiens/Culex torrentium* (Kronefeld et al. 2014) and currently in Slovakia in mosquitoes *Ae. vexans*, indicating the presence of reservoirs and the need for more intensive preventive measures.

Material and methods

Study area

Monitored locations (Ťahanovce—Municipal District Košice, Šebastovce Municipal District—Košice, the river Hornád meander located between Gyňov and Ždaňa, Paňovce, Beniakovce, Košické Olšany villages) are geographically situated in the territory of the Košice Region and spread in the Košice Basin with the total area of 1153 km^2 (Fig. 1). From the climate point of view, the Basin belongs to areas with warm, semi-arid climate with the average annual rainfall of 600–850 mm and 60–70 % (SHMU 2011) air humidity. Almost the entire area is typical for early onset of spring; winters are rather long (52–60 days) with the average daytime temperatures of 18–20 °C. Winters are mild, with average daily temperatures between −3 and −6 °C (SHMU 2011), short, with small number of days with the snow cover. The long-term average daytime annual temperature is 8.7 °C.

Ťahanovce (48° 45′ 22″ S; 21° 15′ 30″ V) It is originally a separate village, today a municipality district of the town of Košice, located on the western edge of the Košice Basin, in the river Hornád basin, in the altitude of 221 m above sea level. Local buildings are of a typical rural type. Mosquitoes were collected on the border between the forest and the gardening area.

Šebastovce (48° 39′ 17.64″ S; 21° 16′ 13.44″ V) It is located in the altitude of 209 m above sea level, originally a separate village, today a municipality district of the town of Košice, of a typical rural nature. In the marginal zone of the village, there is a road leading through Seňa to the Hungarian border crossing. Mosquitoes were collected in adjacent agrocenoses.

Meandres of the river Hornád It spreads from Trstené pri Hornáde to Milhosť. They represent preserved natural remains of a flooded forest with a very rich ecosystem. The monitored area is located between the village of Gyňov (48° 35′ 18″ S; 21° 18′ 15″ V) 206 m above sea level and the village of Ždaňa (48° 36′ 00″ S 21° 20′ 00″ V) 185 m above sea level.

Beniakovce (48° 46′ 10″ S; 21° 19′ 3″ V) The village is located 12 km northeast from Košice, in the altitude of 203 m above sea level. Local buildings are typical family houses. Mosquitoes were collected directly in the village on the private parcel, near a dog breeding facility.

Košické Olšany (48° 44′ 0.96″ S; 21° 20′ 44.52″ V) It is located in the southern part of the Košice Basin, in the river Torysa basin, in the altitude of 202 m above sea level. The collections were carried out on a private parcel behind a family house on a land used for agricultural purposes.

Paňovce (48° 38′ 45.96″ S; 21° 3′ 47.52″ V) It is located in the northwestern part of the Košice Basin, in the altitude of 241 m above sea level. The traps were located near the "Paňovce" pond, Sivé Lake and in the forest.

Mosquito trapping and molecular analysis

Mosquitoes were collected using CO_2-baited traps which were exposed from 5–6 p.m. until 7–8 a.m. of the following day. Mosquitoes were collected in weekly intervals from April to August. Collected individuals were transported from the field in dry ice, stored in the laboratory at −18 °C. Mosquitoes were morphologically diagnosed using available identification keys (Kramař 1958; Becker et al. 2010).

DNA was extracted using 105 pools consisting of 100 individuals of the same species, collected at the same location on the same day. DNA of *Dirofilaria* spp. for the molecular analysis was extracted from whole mosquitoes, by homogenization of the entire pool, using a mallet, in the grinding mortar in 500 μl of PBS. Extracted DNA was isolated using a commercially manufactured set DNeasy® Blood and Tissue Kit (QIAGEN, GmbH., Hilden, Germany) as specified by the manufacturer's instructions.

Genotypization of both dirofilaria species was carried out using primers of known sequences of the cytochrome oxidase gene subunit (COI) specific for *D. immitis* DI COI-F1 (5′-AGT GTA GAG GGT CAG CCT GAG TTA-3′) and DI COI-R1 (5′-ACA GGC ACT GAC AAT ACC AAT-3′) and DR COI-F1 (5′-AGT GTT GAT GGT CAA CCT GAA TTA-3′) and DR COI-R1 (5′-GCC AAA ACA GGA ACA GAT AAA ACT-3′) for D. *repens* (Rishniw et al. 2006). All samples were examined separately for *D. repens* and *D. immitis*.

PCR products obtained after the DNA amplification were then sent to be purified and sequenced in the Laboratory of

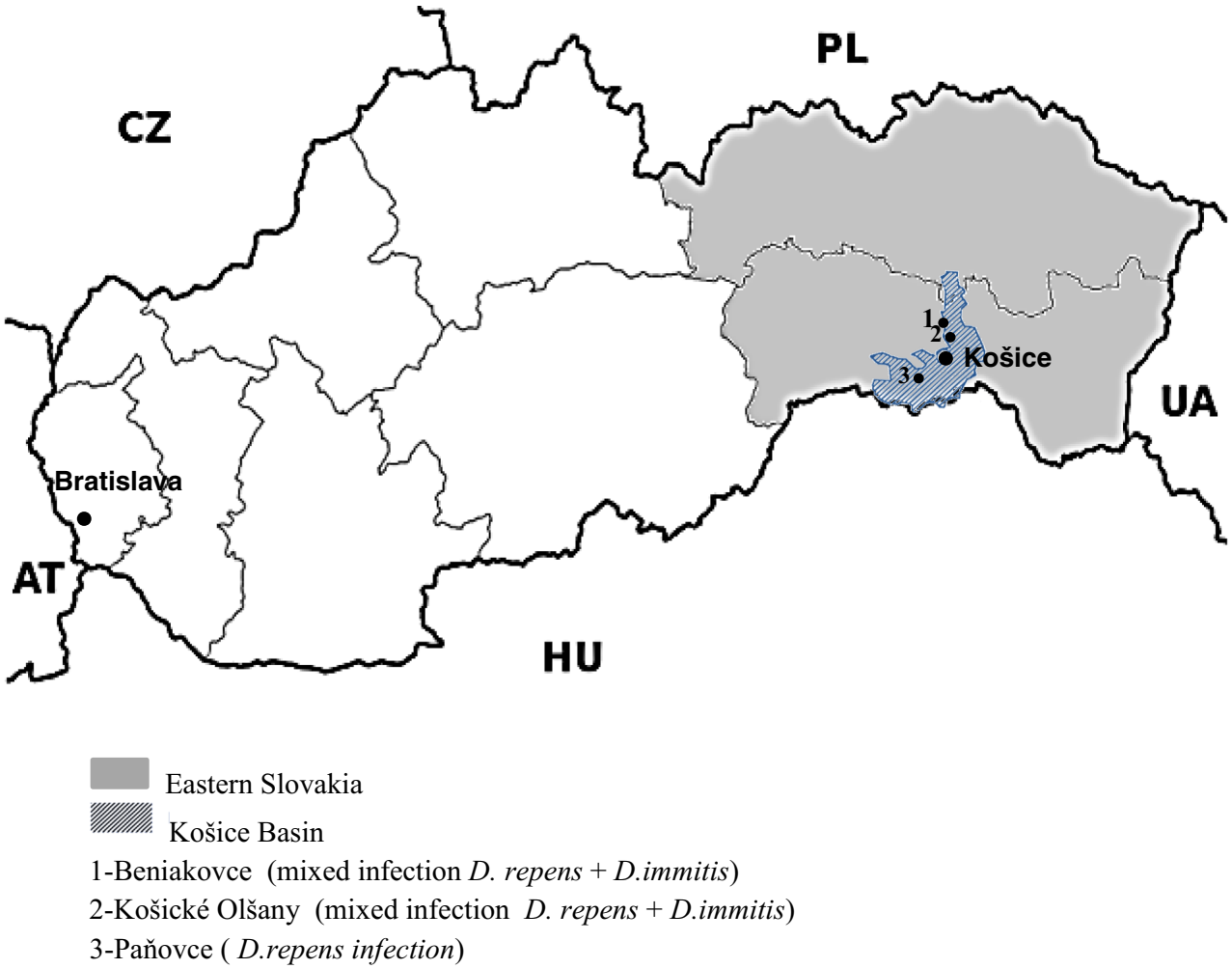

Fig. 1 Locations of Košice Basin area for mosquito trapping in Eastern Slovakia. Points 1, 2, 3 marked sites with positive mosquito findings

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Result and discussion

Ten thousand five hundred mosquitoes were examined for the presence of *D. repens* and *D. immitis* (*Ae. vexans*, *n*=6900; *Ae. rossicus*, *n*=200; *Anopheles maculipennis* sensu lato, *n*=200; *Ochlerotatus sticticus*, *n*=1200; *Ochlerotatus cantans*, n=400; *Ochlerotatus caspius*, *n*=200; *Cx. pipiens/torrentium*, *n*=1200; *Coquillettidia richiardii*, *n*=200), originating from the collections of the 2013 season. Out of 105 samples, there were 6 pools (5.71 %) positive for *Dirofilaria* spp. DNA, consisting of mosquitoes *Ae. vexans*. DNA of *D. repens* was confirmed in all 6 positive pools (5.71 %); out of these, 4 pools (3.81 %) were positive also for *D. immitis* (Table 1, Fig. 2). For the purpose of more transparent electrophoretic visualization, all positive samples were gathered on the common gel; sample no. 17 is negative (Fig. 2). The identity of both filarial species were confirmed by direct sequencing of PCR product, which has shown 99–100 % homology with sequence attributed to *D. repens* and 97–100 % to *D. immitis* (Table 1).

Previous study focused on the molecular screening of *Dirofilaria* spp. DNA in mosquitoes provided the results on the potential vector of *D. repens* in Slovakia (Bocková et al. 2013a, c). Current research discovered not only another area with the incidence of infected mosquitoes *Ae. vexans* (Beniakovce location) but at the same time also a potential risk of transmission of *D. immitis* by this species and possible existence of reservoir.

From the veterinary medicine point of view, *D. immitis* is more important as a causative agent of a cardiopulmonary disease form. In Slovakia, it was detected in dogs in one case as an imported mono-infection and in nine cases in a combination of infections with *D. repens* (Miterpáková et al. 2008; Iglodyová 2014). A prevailing species is still *D. repens* with the 10 % average prevalence in dogs (Iglodyová 2014). In endemic areas (Borská, Danubian, and Eastern Slovak

Table 1 Collection details of *D. immitis* and *D. repens* in *Aedes vexans* mosquito-positive pools

Sample no.	Filarial species	GenBank accession no.	Max. % identity to GeneBank to entry	Collection date	Location
1	*D. repens*	KC 985240.1	99	4/7/2013	Košické Olšany
8	[1]*D. repens* + [2]*D. immitis*	[1]KC 985240.1 [2]AB 973226.1	100 100	19/6/2013	Košické Olšany
43	[1]*D.repens* + [2]*D. immitis*[a]	[1]KC 985240.1 [2]Not assigned[a]	100 –	19/7/2013	Beniakovce
45	*D. repens*	KC 985240.1	99	19/7/2013	Beniakovce
46	[1]*D. repens* + [2]*D. immitis*	[1]KC 985240.1 [2]AB 973226.1	99 100	19/7/2013	Beniakovce
47	[1]*D. repens* + [2]*D. immitis*	[1]KC 985240.1 [2]AB 973226.1	99 97	19/7/2013	Beniakovce

[a] Short sequence

[1,2] mixed infection of *D. repens* and *D. immitis*

Lowlands), it reaches the level of 30 % (Miterpáková et al. 2008; Iglodyová et al. 2012). One of the key factors of the dirofilariosis spread is the ambient temperature which affects not only the geographical distribution of vectors but also the final duration of parasite development inside a mosquito and the disease spread rate. All endemic areas, as well as areas with the increased incidence of canine diseases, are concentrated in a warm climate zone in this territory, where there are, besides suitable conditions for a parasite development, also suitable conditions for vector development and survival (Minář et al. 2007; Strelková and Halgoš 2012; Bocková and Kočišová 2011; Bocková 2013). Lower incidence of diseases in colder northern areas (Poprad, Martin, Žilina) probably relates to less suitable conditions and absence of suitable vectors. Some mosquito species show small-scope specificity for given areas in the territory and, depending on local environmental factors, their population density changes as well. By comparing the species diversity, abundance, and distribution of mosquitoes at selected suburban and urban locations of the Eastern Slovakia, we observed in urban areas as much as 6.5-fold higher concentration of mosquitoes (Bocková 2013). Density of dominant species (*Ae. vexans*, *Cx. pipiens/torrentium*, *and Oc. sticticus*) was 3.3 and 6.28 and 22.8-fold higher than in suburban environment.

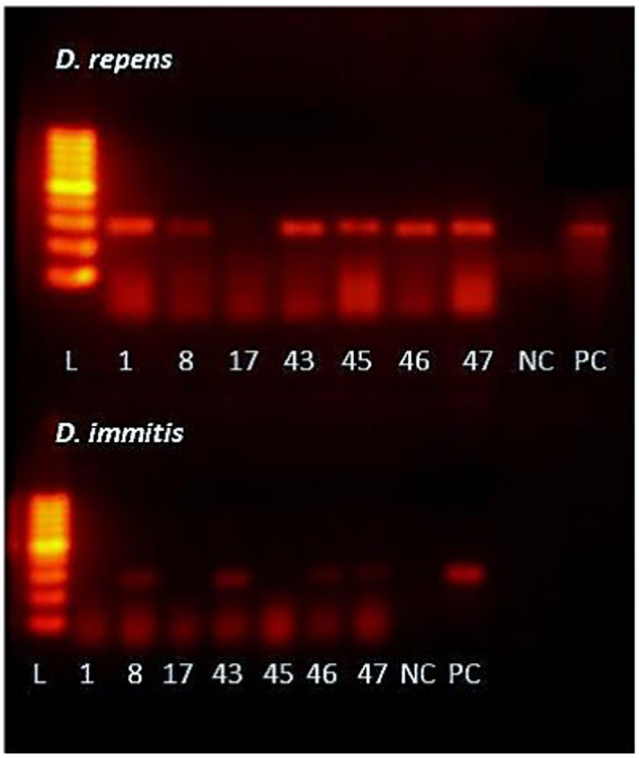

Fig. 2 Gel electrophoresis of the amplified product of the COI gene of *D. repens* and *D. immitis*. *L* ladder, *1*,*8*,*17*,*43*,*45*,*46*,*47* number of pools, *NC* negative control, *PC* positive control

The distance between the two locations where we collected the infected mosquitoes is 8 km (Fig. 1). They are situated in the river Torysa basin, and their biotopes are of a similar nature. In Beniakovce village, mosquitoes were collected in a garden behind a family house, near a dog-breeding facility. In terms of species diversity, in this area, we have observed 11 mosquito species with the dominant representation of *Ae. vexans* (60.10 %), *Cx. pipiens/torrentium* (25.80 %), and *Oc. caspius* (11.20 %).

In Košicke Olšany village, the traps are also located in a garden behind a family house, about 80 m from a water resource. Mosquito species composition includes 13 species. The most represented ones are *Ae. vexans* (77.20 %) and *Cx. pipiens/torrentium* (13.20 %).

None of the listed locations is known for the prevalence of canine dirofilariosis.

With regard to the nature of the surrounding environment (adjacent agrocenoses) and their synanthropic lifestyle, foxes represent an important natural reservoir. In the Eastern Slovakia, the prevalence of *D. repens* in foxes ranges between 53 and 79 % (Hurníková and Miterpáková 2008; Hurníková et al. 2010; Lecová 2011; Iglodyová 2014); *D. immitis* has not been documented so far.

Conclusion

The spread of dirofilariosis is the result of climatic changes which affect Slovakia as well (the average annual temperature increase in 1.7 °C; the average annual rainfall decrease in 0.5 %, relative air humidity decrease). A growing number of autochthonous infections in dogs and humans relates to conditions suitable for vector survival and spread of the mosquito species which did not occur here before (Bocková et al. 2013b). Despite the efforts to inform the lay people and experts about the current situation regarding infections and risks of dirofilariosis, preventive measures still fail, mainly among dog owners. The objective of the follow-up work is to continue with the general entomological and molecular screening of mosquitoes and identify the current rate of filarial infections in vectors.

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