Prevalence of leptospiral antibodies in the red fox (*Vulpes vulpes*) population of Croatia

A. Slavica[1], D. Dezdek[2], D. Konjevic[1], Z. Cvetnic[2], M. Sindicic[1], D. Stanin[1], J. Habus[1], N. Turk[1]

[1]Faculty of Veterinary Medicine University of Zagreb, Zagreb, Croatia
[2]Croatian Veterinary Institute, Zagreb, Croatia

ABSTRACT: Over a period of five years (2005–2010) a total of 358 red fox (*Vulpes vulpes*) serum samples were collected during regular hunting procedures, mostly in the continental part of Croatia. All samples were tested using the microscopic agglutination test (MAT) for the presence of specific antibodies (Ab) to 12 *Leptospira* spp. pathogenic serovars. Specific antibodies for 11 different *Leptospira* spp. serovars (Australis, Sejroe, Icterohaemorrhagiae, Saxkoebing, Grippotyphosa, Tarassovi, Ballum, Pomona, Poi, Batavie, and Canicola) were detected in 121 red fox serum samples (33.8%). Among the positive samples antibodies for serovar Australis were found most frequently (32.1%), followed by Abs for serovar Sejroe (18.2%) and Icterohaemorrhagiae (13.2%). The highest Ab titre (1 : 3200) was recorded for serovar Australis and together with serovars Sejroe and Icterohaemorrhagiae this serovar showed a statistically significant frequency in cross-reactions (recorded in 23.1% cases). High Ab titres for serovars Grippotyphosa and Tarassovi (1 : 800) were detected for the first time in red foxes from the continental part of Croatia. The significant percentage (≈ 35%) of seropositive reactions to *Leptospira* spp. serovars implies the presence of pathogenic agents in the natural habitats of red foxes in Croatian low-land territories. The high Ab prevalence for specific serovars (Australis and Sejroe) detected in the fox population suggests predator-prey chain transmission of leptospirosis between red foxes and small terrestrial mammals, whilst the high Ab prevalence for serovar Icterohaemorrhagiae indicates an occasional contact of red foxes with rats. Red foxes can be thought of as susceptible hosts for serovars Australis and Sejroe and a possible link in leptospirosis transmission to other wild carnivores such as martens, weasels and ferrets. In contrast, this serologic survey revealed no evidence of serovar Canicola interchange between domestic carnivores (dogs) and red foxes. It can be concluded that red foxes are reliable sentinels for the epidemiological monitoring of leptospirosis, especially in lowland habitats.

Keywords: red fox; *Leptospira* spp.; serology; Croatia

List of abbrevations

Ab = antibodies; **F** = female; **M** = male; **MAT** = microscopic agglutination test

As a part of a wildlife health monitoring program a serosurvey of the prevalence of different *Leptospira* spp. serovars in the red fox population of Croatia was carried out. Leptospirosis is a widespread zoonosis with a broad spectrum of clinical presentations and outcomes, caused by spiral Gram-negative bacteria from the genus *Leptospira* (Langston and Heuter, 2003). The genus *Leptospira* is divided into 20 species based on DNA hybridization studies (Bharti et al., 2003) and these 20 species are classified into more than 280 serovars, according to their antigenic relatedness (Cerqueira and Picardeau, 2009). Worldwide distribution of different *Leptospira* serovars has been well documented over the last 50 years, while recently the importance of identifiying prevalent serovars at the regional level has been stressed by numerous authors (Treml et al., 2002; Richardson and Gauthier, 2003; Slavica et al., 2008; Espi et al., 2010).

In the lowland areas of Croatia the first report of leptospiral antibodies in red foxes was published

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44 years ago (Zaharija and Todorovic, 1966), followed by reports by Kovacic et al. (1985) and Milas et al. (2006). All authors reported the presence of different *Leptospira* serovars with antibody prevalence ranging from 11.2% (Zaharija and Todorovic, 1966) to 57.6% (Milas et al., 2006). The specific characteristics of red fox life cycle, namely high reproductive potential, migration of young individuals and opportunistic food habits identifies the red fox as an epizootiologically important species in the chain of leptospirosis transmission. Thus, the aim of this study was to probe the presence of pathogenic *Leptospira* serovars in the red fox population of Croatia and to research the role of this species in the maintenance of leptospirosis in the continental regions of Croatia.

MATERIAL AND METHODS

From February 2005 to January 2010 blood samples from 358 red foxes were collected during the regular hunting season. Samples originated from 181 male (M) and 177 female (F) animals of various ages and body weights. The age of sampled red foxes was estimated by analysing the cementum annuli in the hard tooth tissues (Harris, 1978), particularly in the separation of the young-of-the-year from adults. We also used a simple method of fox age determination described by Roulichova and Andera (2007). Studied red foxes were classified based on age as juveniles (< 1 year old) and adults (> 1). The weight of the sampled animals was measured to the nearest 0.1 kg (Tryjanowski et al., 2009).

Samples were collected at 37 different locations from the continental part of Croatia and the sampling area was divided into six main regions: (1) Podravina region (Varazdin, Ludbreg, Koprivnica, Durdevac, Virovitica, Podravska Slatina); (2) Zagorje region (Ivanec, Krapina, Zlatar, Zabok, Sv. Ivan Zelina, Marija Bistrica); (3) Posavina region (Dugo Selo, Ivanic Grad, Sisak, Kutina, Novska, Nova Gradiska, Slavonski Brod); (4) Kordun region (Duga Resa, Bosiljevo, Karlovac, Slunj, Vojnic); (5) Gorski Kotar region (Vrbovsko, Ogulin, Mrkopalj, Fuzine, Delnice); (6) Slavonija and Baranja region (Valpovo, Beli Manastir, Tikves, Knezevo, Osijek, Dakovo, Vinkovci, Zupanja).

The collection of blood samples was performed aseptically, directly from the heart of shot foxes using a long needle (Poljicak-Milas et al., 2004) using a modification of the method described by Slavica et al. (2000). Blood samples were stored in serum separator tubes, centrifuged at 2200 rpm and transported to a laboratory within 12 hours. All sera were tested by microscopic agglutination test (MAT) for the presence of 12 leptospiral antigens. The MAT was performed according to standard methods (Faine, 1982) and used strains, together with investigated serovars and *Leptospira* species, are listed in Table 1. An initial sera dilution of 1 : 100 was considered as a cut-off point for a positive reaction. The differences between positive reactions and the number of positive samples were noted, due to the high possibilities of multiple co-agglutinations (cross-reactions) among several antigens of *Leptospira* (Milas et al., 2002). For data analysis the standard statistical package Sigma Stat for Windows (SPSS/PC Version 3.0) was used. *P* values of <0.05 were considered significant.

Table 1. Species of *Leptospira* spp., related serovars and strains used in the MAT procedure

Species	Serovar	Strain
L. borgpetersenii	Ballum	Mus 127
L. borgpetersenii	Poi	Poi
L. borgpetersenii	Sejroe	M 84
L. borgpetersenii	Tarassovi	Mitis Johnson
L. interrogans	Australis	Ballico
L. interrogans	Bataviae	Van Tienen
L. interrogans	Canicola	Hond Utrecht IV
L. interrogans	Hardjo	Hardjoprajitno
L. interrogans	Icterohaemorrhagiae	RGA
L. interrogans	Pomona	Pomona
L. interrogans	Saxkoebing	Mus 24
L. kirshneri	Grippothyphosa	Moskva V

RESULTS

The results of MAT revealed the presence of antibodies (Ab) for 11 different *Leptospira* spp. serovars in 121 of 358 (33.8%) red fox sera, with titres ranging from 1 : 100 to 1 : 3200 (Table 2). In total we recorded 159 positive reactions, and in 28 (23.1%) out of 121 positive sera we detected Abs for more than one serovar – ranging from two to five serovars per sample. Out of all positive reactions we found the highest Ab prevalence for serovar Australis (51/159, 32.1%), followed by serovar Sejroe (29/159, 18.2%) and serovar Icterohaemorrhagiae (21/159, 13.2%). Serovar Australis also showed the highest

Table 2. *Leptospira* spp. serovars tested by MAT with percentage of positive reactions and titres in red foxes

Leptospira spp. serovars	Positive reactions		Titre					
	n	%	100	200	400	800	1600	3200
Australis	51	32.1	34	6	5	2	3	1
Sejroe	29	18.2	20	4	2	2	1	–
Icterohaemorrhagiae	21	13.2	14	3	2	1	1	–
Saxkoebing	17	10.7	11	3	3	–	–	–
Grippothyphosa	12	7.5	7	2	2	1	–	–
Tarassovi	10	6.3	6	2	1	1	–	–
Ballum	8	5.0	5	2	1	–	–	–
Pomona	5	3.2	4	1	–	–	–	–
Poi	3	1.9	2	1	–	–	–	–
Bataviae	2	1.3	1	1	–	–	–	–
Canicola	1	0.6	1	–	–	–	–	–
Hardjo	0	0	0	0	0	0	0	0
Total	159		105	25	16	7	5	1
Percent	100	100	66.05	15.7	10.1	4.4	3.15	0.6

Ab titre (1 : 3200), as well as the highest percentage of cross-reactions (41.5%), mostly with serovars Sejroe, Icterohaemorrhagiae and Saxkoebing. High Ab titres for serovars Grippotyphosa and Tarassovi (1 : 800) were detected for the first time in red foxes from the continental part of Croatia. No positive serological reactions were recorded for serovar Hardjo, although we found one sera with a very low serological reaction to serovar Hardjo (titre 1 : 50). We observed no significant differences ($P > 0.05$) with respect to age and sex in Ab prevalence between adults (54.6%) and juveniles (45.4%), or male (M – 47.5%) and female (F – 52.5%): specimens. With respect to geographical distribution the highest percentage of positive red fox samples originated from lowland habitats in the Posavina region (Ivanic Grad – 47.1%) and Zagorje region (Sv. Ivan Zelina – 46.9%), while the lowest percentage of positive red foxes was recorded in the Gorski Kotar region (Mrkopalj 11% and Fuzine 7%).

DISCUSSION

Research on fox *Leptospira* spp. seroprevalence requires comprehensive ecological knowledge of fox population dynamics and must include considerations of juvenile fall migrations, home range, population density, litter size, yearly accession, mortality rate and hunting pressure (Milas et al., 2006). Epidemiological data of leptospiral archaic foci, reservoirs, maintenance hosts and serovar distribution are also very important (Slavica et al., 2008). The results of our survey confirmed the presence of specific Abs to different serovars of *Leptospira* in the red fox population in most regions of the Croatian territory. The prevalence of Abs against leptospiral serovars found in this study (33.8%) was lower than the prevalence reported in red foxes from Spain (47.1%; Millan et al., 2009) and red foxes from northwest Croatia (57.6%; Milas et al., 2006), while it was slightly higher than the prevalence recorded five years ago in the Croatian red fox population (31.25%; Slavica et al., 2008).

The most frequently detected serovars in the present sero-survey were Australis and Sejroe (which have rodents as main reservoirs), followed by serovar Icterohaemorrhagiae (which has rats as main reservoir). Predator-prey chain transmission was suggested (Reilly et al., 1970) as a possible natural route of passing leptospiral infections from rodents to red foxes, and other free-living carnivores (Hathaway et al., 1983). In the continental part of Croatia the yellow-necked field mouse (*Apodemus flavicolis*) was postulated to be the main reservoir of serovar Australis (Milas et al., 2002), whilst the striped field mouse (*Apodemus agrarius*) together with the wood mouse (*Apodemus sylvaticus*) were identified as reservoirs for serovar Sejroe. Turk et al. (2003) isolated *Leptospira* spp. serogroup Sejroe from domestic

mice (*Mus musculus*), serogroup Australis from yellow-necked field mice and serogroup Pomona from striped field mice. All these rodent species are important food sources for foxes, especially in the autumn time, when small mammals represent almost 40% of the fox diet (Milas et al., 2006). The high prevalence of serovars Australis (32.1%) and Sejroe (18.2%) found in this survey confirm the contact of foxes with mouse-like rodents, while the high prevalence of Abs for serovar Icterohaemorrhagiae (13.2%) implies a contact of foxes with rats (*Rattus norvegicus*), presumably at garbage dumps and other waste disposals. Rats are acknowledged to be the main reservoirs of serovar Icterohaemorrhagiae in Croatia (Zaharija and Todorovic, 1966) and they were identified as a possible source of *Leptospira* serovars for captured brown bears (*Ursus arctos*) from Zagreb Zoo (Modric and Huber, 1993) and free-ranging bears from the Lika and Gorski Kotar regions (Slavica et al., 2010a). During the last decade brown bears, like red foxes have changed their feeding habits and often visit waste disposals and feeding stations, where the high concentration of rats enhances the chances of 'prey to predator' transmission of serovar Icterohaemorrhagiae.

The importance and host range of serogroup Australis in Europe was stressed for the first time by Hathaway et al. (1983) and after that serovars from the Australis serogroup were found in different domestic (Ellis et al., 1986, 1991; Burriel et al., 2003) and wildlife species (Wilson et al., 1998; Milas et al., 2002, 2006; Turk et al., 2003; Slavica et al., 2008; Millan et al., 2009). In Croatia serovars from the Australis serogrup were detected for the first time more than 50 years ago by Zaharija (Milas et al., 2002) and were mostly represented by serovars Australis and Lora (Turk et al., 2003). Serovar Australis has been described to be especially predominant in wild boars (Slavica et al., 2008, 2010b) and red foxes (Milas et al., 2006; Slavica et al., 2008), the only two wild species in Croatia for which the population density has remained stable or even risen. In the last decade the shift of red foxes from rural to suburban and urban habitats in the continental parts of country has increased the possibility of their cohabitation with domestic carnivores, especially with free-roaming dogs. Stray dogs are usually not vaccinated against leptospirosis and could be infected easily by different *Leptospira* serovars. In our study red foxes showed very low Ab titres for serovar Canicola the main natural reservoir of which is the dog, but recent serological research showed an increasing prevalence of serovars Australis (Habus et al., 2008) and Icterohaemorrhagiae (Modric et al., 2006) in the Croatian dog population.

In conclusion, the results of our survey demonstrate that interactions between different *Leptospira* serovars are frequent in the red fox population in continental parts of Croatia, especially in lowland terrains with abundant water and a high density of myomorphous mammals. In order to determine the role of red foxes in the transmission of leptospirosis the isolation of *Leptospira* spp. from kidney is critical, and in our future research we will attempt to identify fox urine as a possible source of leptospiral infections for wild and domestic carnivores, livestock and humans.

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Corresponding Author:

Alen Slavica, PhD, DVM, University of Zagreb, Faculty of Veterinary Medicine, Department of Game Biology, Pathology and Breeding, Heinzelova 55, 10 000 Zagreb, Croatia
Tel. +385 1 2390 156, E-mail: slavica@vef.hr