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Serologic Evidence of *Leptospira* spp. Serovars in Brown Bears (*Ursus arctos*) from Croatia

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ABSTRACT: Serum samples from 52 free-ranging brown bears (*Ursus arctos*) collected in Croatia over a period of 10 yr (1998–2007) were tested by microscopic agglutination test for specific antibodies (Ab) to 12 *Leptospira* spp. pathogenic serovars. At titers ranging from 1:100 to 1:2,000, 19 samples (36.5%) were Ab-positive to at least one serovar. Antibodies for 10 *Leptospira* spp. serovars were detected: Icterohaemorrhagiae, Australis, Sejroe, Canicola, Poi, Hardjo, Ballum, Saxkoebing, Pomona, and Grippotyphosa. In comparison to previous reports, the prevalence of Ab to serovar Icterohaemorrhagiae (52.6%) was significantly higher. Other common serovars were Australis (47.4%) and Sejroe (42.1%). High Ab titers for serovars Canicola (1:500) and Grippotyphosa (1:1,000) were detected for the first time in free-ranging bears from Croatia. A significant correlation between the age of the bears and detection of Ab to *Leptospira* spp. serovars suggested the presence of pathogenic agents in the natural habitats, whereas increasing trends of Ab prevalence for specific serovars (Icterohaemorrhagiae, Australis, and Sejroe) confirmed cohabitation of bears with rats and other small terrestrial mammals on garbage dumps and at bear feeding stations. To prevent cohabitation of bears and rodents, improvements in Croatian waste treatment, big game management, and rodent control programs are strongly recommended, especially in Lika and Gorski Kotar, regions that have high-quality natural habitats for brown bears in Croatia.

Key words: Brown bear, Croatia, *Leptospira* spp., rodents, serology, *Ursus arctos*.

Leptospirosis is one of the world's most common zoonoses. Infectious strains of *Leptospira* spp. are transmitted in nature primarily via water contaminated with urine of various wild mammals. Although infection by pathogenic *Leptospira* serovars is common in a wide range of wildlife hosts, rodents have been considered as the most important reservoirs (Milas et al., 2002; Turk et al., 2003). In Croatia, serologic surveys of *Leptospira* serovars in wildlife began 25 yr ago, first on small terrestrial mammals (Borčić et al., 1982) and later on other epidemiologically interesting species (Kovačić et al., 1984; Borčić et al., 1989). Since 1985, three serologic surveys have been carried out in brown bears (*Ursus arctos*) in the Gorski Kotar and Lika regions. All surveys revealed the presence of antibodies (Ab) to *Leptospira* serovars with Ab prevalences ranging from 20% to 40% (Karlović et al., 1985, 1990; Modrić and Huber, 1993).

Brown bears in Croatia are part of the larger Dinaric population, estimated to be approximately 2,800 bears for the entire Dinaric and Pindos mountain area (Huber et al., 2008b). This is the second largest population in Central and Southern Europe (Fig. 1). The total bear distribution area in Croatia encompasses 11,825 km^2, which is inhabited by 600 to 1,000 bears (Huber et al., 2008a). The best habitats—Gorski Kotar (45°27′N, 14°38′E), Velika Kapela (45°13′N, 14°58′E), Mala Kapela (44°58′N, 15°24′E), and Velebit (44°48′N, 15°11′E)—support an average density of one to two bears per 10 km^2 (Kusak and Huber, 1998). Brown bears are omnivores; their diet consists of up to 95% plant material and they exhibit distinct seasonal cycles in diet (Cicnjak et al., 1987). To satisfy an increasing energy requirement

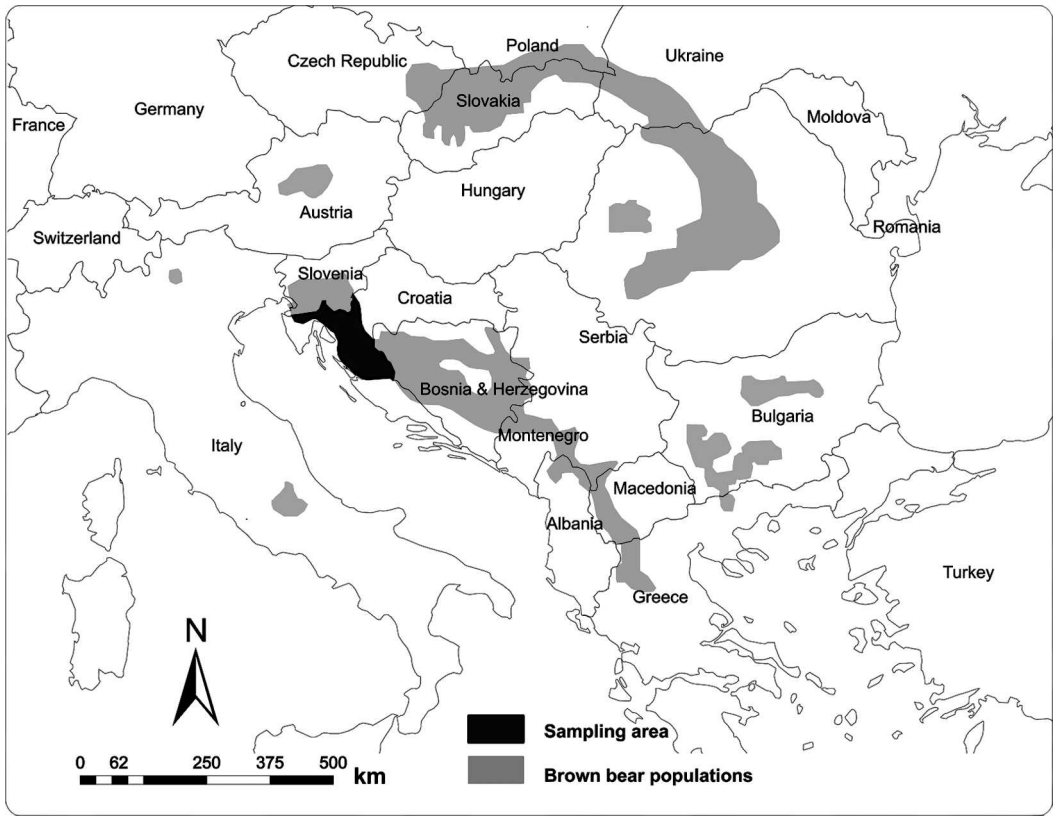

FIGURE 1. Study area and range of brown bear (*Ursus arctos*) populations in south-central Europe (according to Huber et al., 2008b).

in the autumn, bears feed in lower altitude areas and near human settlements, usually foraging during the night (Huber et al., 2008a). This nocturnal activity pattern, which is exhibited by a majority of adult brown bears in Croatia and Slovenia (Kaczensky et al., 2005) provides the opportunity for bears to forage in garbage dumps, where a high probability of contact between bears and rodents could increase the risk of *Leptospira* transmission. The objectives of our serologic survey were to determine the Ab prevalence of different serovars of *Leptospira* spp. in brown bears in relation to their geographic origin, age, sex, and food habits, as well as to identify possible sources of leptospiral infection for bears in Croatia.

Over the 10 yr (1998–2007), we collected 52 blood samples of brown bears (39 male and 13 female) of various ages and body weights. Twenty-four samples originated from the Lika region (44°58′N, 15°24′E) and 28 were from Gorski Kotar. Age of sampled bears was estimated by examining the cementum layers on the root of the extracted first premolar (Stonenberg and Jonkel, 1966). Cubs were defined as bears under the age of 1.5 yr, when they normally separate from the mother. Most sexually mature female bears in Croatia have a litter every second year (Frković et al., 2001), giving birth during the denning period. All samples were taken from free-ranging bears; 34 were collected from bears shot by hunters and 18 were taken from bears that were chemically immobilized (11 mg/kg of ketamine hydrochloride; Ketalar®, Parke-Davis, Berlin, Germany, and 6 mg/kg of xylazine hydrochloride; Rompun®, Bayer, Leverkusen, Germany) for radio-tagging.

From immobilized bears, blood was drawn from the femoral vein (Modrić and Huber, 1993) using vacuum collection tubes (Becton–Dickinson, Meyland Cedex, France); from shot bears blood was taken from the heart using a syringe and long needle (Slavica et al., 2000). Blood samples were transported to the laboratory within 12 hr and sera were tested by microscopic agglutination test (MAT; Gochenour et al., 1958) for 12 *Leptospira* antigens (serovars Icterohaemorrhagiae, Australis, Sejroe, Canicola, Poi, Hardjo, Ballum, Saxkoebing, Grippotyphosa, Tarassovi, Bataviae, and Pomona). Serum samples that were Ab-positive at the initial dilution (1:100) for a given serovar were examined up to the endpoint of 50% agglutination (maximum dilution). Because of the high cross-reactivity among *Leptospira* antigens (Krawczyk, 2005), we recorded the differences in endpoint titers among serovars for each sample (Modrić et al., 1985). Samples with ≥2 serovars with titers ≥1:500 were considered as sera with multiple serovars. If one sample was positive for two or more *Leptospira* antigens, the serovar with highest titer was considered as the earlier infection (Milas et al., 2006). Results were analyzed (data distribution, Pearson's chi-square test) using Sigma Stat for Windows Version 3.0 (SPSS, Inc). The value of $P<0.05$ was considered statistically significant.

We found antibodies for 10 *Leptospira* spp. serovars in 19 (36%) of 52 brown bear sera, with titers ranging from 1:100 to 1:2,000 (Table 1). Nine positive samples originated from Lika (47%) and 10 were from Gorski Kotar (53%). The majority ($n=39$) of endpoint reactions ($n=57$) were at 1:100 (68%), 14 (25%) were at 1:500, three (5%) were at 1:1,000, and one (2%) was at 1:2,000. In 16 (31%) of 52 sera, we detected Ab to more than one serovar—ranging from two to five serovars per sample. Multiple serovars (titers ≥1:500) were detected in 11 sera (21.1%), mostly among serovars Icterohaemorrhagiae, Australis, and Canicola. Antibody titers to serovars Canicola (1:500) and Grippotyphosa (1:1,000) were the highest ever detected to these serovars in free-ranging bears from Croatia. The highest Ab prevalence was for serovar Icterohaemorrhagiae, for which we found specific Ab in 10 (52%) of the 19 Ab-positive bear samples. Serovar Icterohaemorrhagiae also had the highest Ab titer (1:2,000), as well as a high percentage of cross-reactions (31%) and was the serovar that most frequently had the highest titer for samples that reacted to more than one serovar. In comparison to past surveys (Karlović et al., 1985; Modrić and Huber, 1993), the bears we tested had a significantly higher prevalence of Ab to serovar Icterohaemorrhagie ($P<0.0001$, Pearson's chi-square test; Fig. 2). Serovars Australis (47%) and Sejroe (42%) also showed strong Ab reactions, but in comparison to past surveys we recorded smaller increases in the percentage of samples with Ab to those two serovars. There was no significant difference ($P>0.05$) in Ab prevalences between the bears from Lika (nine positive of 24 samples examined) and Gorski Kotar (10 of 28). There was no significant difference ($P>0.05$) in prevalence among genders (four of 13 females and 15 of 39 males), but there was a highly significant difference in prevalence between cubs and older bears ($P<0.001$).

All seven blood samples from bear cubs were negative. This result is in accordance with findings of Modrić and Huber (1993), who found six Ab-negative bear cubs in Gorski Kotar, and one positive cub in Lika with very low serologic reactivity (serovar Sejroe, titer 1:100). The youngest Ab-positive bear in our survey was a 2-yr-old female from Gorski Kotar, which had a clear positive reaction to serovars Canicola and Pomona at initial dilution, and was borderline positive for serovar Batavie (Table 1). Another two young females (age 3 yr) were positive for serovars Australis and Sejroe. The most frequent serovar, Icterohaemorrhagie, was found only in older bears (≥4 yr). The low

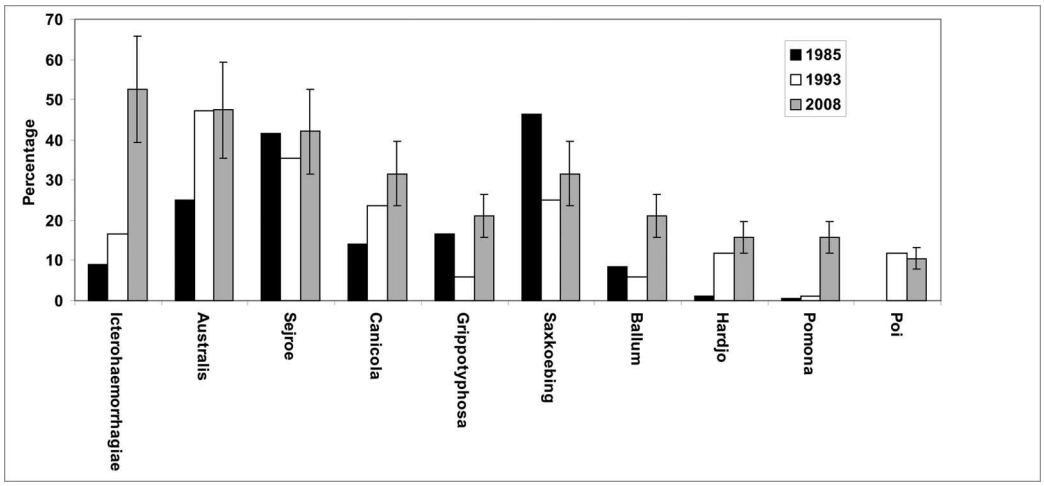

FIGURE 2. Percentage of samples with antibody to *Leptospira* spp. serovars for brown bears (*Ursus arctos*) from Croatia during three surveys (1985, 1993, and 2008). Calculations were done exclusively on antibody-positive samples.

TABLE 1. Reciprocal titers of *Leptospira* spp. antibodies in 19 brown bear sera, with frequency of the highest titer.

Bear status[a]	Sex[b]	Age (yr)	*Leptospira* serovars[c]											
			Icter	Aus	Sej	Can	Gri	Sax	Bal	Bat	Har	Tar	Pom	Poi
TL1	F	3	—	—	100	—	—	—	—	—	—	—	—	—
HL1	M	5	100	—	—	500	—	100	—	—	—	—	100	—
TL2	F	3	—	100	—	—	—	—	—	—	—	—	—	—
HL2	M	7	100	500	—	100	1,000	—	—	—	—	—	—	—
HL4	M	4	—	100	100	—	—	100	—	—	—	—	—	—
HL5	M	5	100	—	500	100	—	—	—	—	—	—	—	—
HL6	M	8	500	100	—	—	—	100	—	—	—	—	—	100
HL7	M	6	100	500	—	—	—	—	—	—	—	—	—	100
HL8	M	4	—	—	100	—	—	500	—	—	100	—	—	—
TG1	M	3	—	—	100	—	100	—	100	—	—	—	—	—
HG1	F	5	500	100	—	100	—	—	—	—	—	—	—	—
TG2	M	4	—	—	—	—	100	—	—	—	—	100[d]	100	—
HG2	M	7	2,000	1,000	500	—	—	500	—	—	1,000	—	—	—
TG3	F	2	—	—	—	100	—	—	—	100[d]	—	—	100	—
HG3	M	6	500	100	—	100	—	—	—	—	100	—	—	—
HG4	M	5	100	—	500	—	—	500	500	—	—	—	—	—
HG5	M	4	—	—	100	—	100	—	100	—	—	—	—	—
HG6	M	4	—	—	—	—	—	—	100	—	—	—	—	—
HG7	M	5	500	100	—	—	—	—	—	—	—	—	—	—
Frequency of HT[e]			5	2	2	1	1	1	1	—	—	—	—	—

[a] TL = tagged Lika; HL = hunted Lika; TG = tagged Gorski Kotar; HG = hunted Gorski Kotar.

[b] F = female; M = male.

[c] Icter = Icterohaemorrhagiae; Aus = Australis; Sej = Sejroe; Can = Canicola; Gri = Grippotyphosa; Sax = Saxkoebing; Bal = Ballum; Bat = Batavie; Har = Hardjo; Tar = Tarassovi; Pom = Pomona; Poi = Poi.

[d] Borderline positive.

[e] HT = highest titer.

prevalence of specific leptospiral Ab in bear cubs suggests that they may be protected by nonspecific maternal Ab in early life. Alternatively, due to their young age they would have experienced less exposure to pathogens in the environment.

It has been documented on an international level that brown bears frequently use garbage dumps as a food source (Peirce and Van Daele, 2006). In a study of Croatian brown bear food habits, where hunted food was distinguished from food from garbage dumps, small mammal remains constituted 3% of bear scat samples (Cicnjak et al., 1987). In the Lika and Gorski Kotar regions, adult bears search for food before the hibernation period in extended day and night foraging at large areas that include garbage dumps and bear feeding stations (selected sites where bears are provided with supplemental food during the critical period before hibernation). Rodents are known to be abundant at these sites (Margaletić, 2004). The high prevalence of Ab to serovar Icterohaemorrhagiae (53%) found in this study, together with similar results in recent research (Slavica et al., 2008), suggests that bears have contact with rats (*Rattus norvegicus*) and their excreta at waste disposal sites and at bear feeding stations. Rats, which have been identified as the main reservoirs of serovar Icterohaemorrhagiae in Croatia (Zaharija and Perić, 1969) and are known as lifetime carriers of pathogenic *Leptospira* serovars (Borčić et al., 1982), are a serious public health problem in rural regions of Lika and Gorski Kotar (Margaletić and Kišasondi, 2007). Strong serologic reactions to serovars Australis and Sejroe suggest bears have contact with other small terrestrial mammals (*Apodemus sylvaticus*, *Myodes* [*Clethrionomys*] *glareolus*, *Apodemus flavicollis*), which are carriers of these two serovars in continental parts of Croatia (Borčić et al., 1982). Although we did not find clinical signs of leptospirosis in sampled brown bears, the high prevalence of specific Ab for *Leptospira* spp. suggests that bears could be an accidental host for serovars Icterohaemorrhagiae, Australis, and Sejroe. To minimize contact of bears with rats and other small terrestrial mammals, we support intensified rodent control programs (Margaletić and Kišasondi, 2007) using measures compatible with protection of native wildlife, together with a national brown bear management plan (Huber et al., 2008a, b). Application of management tools (electric fencing, use of bear-proof garbage containers, and disturbing noises or repellents) for prevention of bears feeding on garbage in Lika and Gorski Kotar would help to reduce the risk of *Leptospira* transmission between rodents and bears.

In conclusion, more pronounced synanthropic behavior of brown bears in Lika and Gorski Kotar increases the prevalence of Ab to specific *Leptospira* spp. serovars, especially in adult bears. Although our results demonstrate the presence of pathogenic leptospiral serovars in bear habitat, it will be necessary to isolate and identify the infectious agents in order to positively establish that brown bears from Croatia are accidental hosts for serovars Icterohaemorrhagiae, Australis, and Sejroe.

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