A serological survey and isolation of leptospires from small rodents and wild boars in the Republic of Croatia

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ABSTRACT: In total the samples of blood and kidneys of 379 small rodents and 154 wild swine were analysed. The antibodies to different serovars of leptospires were determined in 12.7% of small rodents, most often in the species *Mus musculus* (34.4%), *A. agrestis* (14.8%), *A. flavicolis* (10.8%), *C. glareolus* (9.4%) and *A. sylvaticus* (6.5%). Most frequent were the findings of antibodies to sv. *pomona* (27.1%), sv. *sejroe* (20.8%), and sv. *australis* (14.6%), and the antibodies to sv. *hardjo*, sv. *saxkoebing*, sv. *tarassovi*, sv. *grippotyphosa*, sv. *bataviae* and sv. *icterohaemorrhagiae* were also established. Seventeen (4.5%) isolates were identified, belonging to the serogroups *sejroe* (10 isolates), *pomona* (4 isolates) and *australis* (1 isolate) and one isolate was not identified. In wild swine positive reactions were established in 26% of the blood sera analysed. Most frequently the antibodies to sv. *pomona* (47.5%), sv. *australis* (40%), sv. *grippotyphosa* (10%) and sv. *icterohaemorhagiae* (2.5%) were established. Thirteen (8.4%) isolates belonging to the serogroups *pomona* (10 isolates), *australis* (2 isolates) and *icterohaemorhagiae* (1 isolate) were identified.

Keywords: leptospirosis; small rodents; wild boars; prevalence; Croatia

Leptospirosis is an acute septicemical infective disease of different kinds of domestic and wild animals and humans (zoonosis) that is caused by different serovars (sv.) of leptospires within the species *Leptospira interrogans*. Small rodents are natural reservoirs of leptospires, and can be their carriers for lifetime. Leptospirosis is a mild, rarely severe clinical disease in pigs and young swine. In pregnant sows it can cause abortions and production of stillborn or weak pigs with reduced viability. Since leptospires are shed by the urine of infected animals, small rodents and wild boars contaminate the environment, grass, surface waters, muddy and swampy areas where leptospires survive, and this contaminated environment becomes a source of leptospires for other animals as well as humans.

In the context of a complex investigation of the ecology of natural foci of leptospires in Croatia, a special attention was paid to micelike mammals as the primary reservoirs of leptospires in nature (Borcic *et al.,* 1978). Borcic *et al.* (1982, 1983) determined rodents as reservoirs of leptospires in the valleys of the rivers Sava and Drava.

There are several studies on the prevalence of leptospirosis in wild boars in Croatia. Kovacic *et al.* (1984) established 9.2% of positive wild boars in the region of Baranja. Borcic *et al.* (1989) described the findings of positive reactions in 11.6% of wild boars in northern Croatia. Kovacic *et al.* (2001) reported about the findings of positive reactions in 6.2% of wild boars in the region of Gorski Kotar. Cvetnic *et al.* (2002) by their investigations in the region of Turopolje established the presence of identical leptospira serovars (sv. *australis* and sv. *pomona*) in rodents and Turopolje swine from the same region.

Preliminary results of the molecular characterization of some of the strains of leptospires analysed in this study were published earlier (Turk *et al.*, 2003). In this paper, the prevalence of different serovars of leptospires in different species of small rodents and wild boars on several localities in the Republic of Croatia has been established.

MATERIAL AND METHODS

Sampling animal population

During 2000, 2001 and 2002 the sampling of rodents and wild boars was carried out and blood samples and kidneys were collected for serological and bacteriological analysis for the presence of leptospirosis. The sampling of animals took place on the territory of the forestry offices of Velika Gorica, Sisak, Kutina, Nova Gradiska, Vrbanja, Gunja and Otok (Figure 1). Microlocalities were chosen in such a way that different biotypes were represented (forest, bushes, meadows, out-skirts of plough-fields or swamps), to get as accurate picture of living terriofauna on a particular locality as possible.

During the investigations on all of the localities in total 445 rodents were caught. Out of that number 379 (85.17%) animals were analysed in a laboratory. The rest of the animals (66) were not tested due to the damage made by predators or decomposition of tissues caused by high temperatures. During the same period, the samples of blood and kidneys of 154 wild boars were also analysed.

For sampling the small rodents traps which kill an animal and traps which catch an animal alive of "Sherman" type were used (Baumler and Brunner, 1988; Margaletic, 1998.). The traps were placed in hunting transection. Apples, oat flakes and sardines in oil were used as baits. The determination of individual animals sampled was carried out according to Niethammer and Krapp (1978; 1982).

Serological testing

Serum samples. In total the samples of 379 rodents and 154 wild swine were serologically analysed. The blood samples of rodents were taken shortly after hunting and the method of dried blood patches on a filter paper was applied. The filter paper of 1 × 5 cm in size was dipped into a thoracic cavity or cut heart and left for blood to penetrate in the paper (the blood should penetrate in 2/3 or 1/2 of the filter paper). It was left to get dried and then stored in small bags (Sebek, 1964). The blood of wild swine after hunting was taken with a syringe from the heart or thoracic cavity and centrifuged already in the field. The sera were stored at –20°C and kept there until delivering to the laboratory for leptospirosis in the Croatian Veterinary Institute in Zagreb.

Serological test. For demonstrating the antibodies to leptospirosis the test of microscopic agglutination was used (Sebek, 1964; Johnson, 1976; Trbic, 1984). For serological analyses of blood samples of small rodents the filter papers with blood were put into the test tubes with 1 ml of physiological solution

Figure 1. Localities in the Republic of Croatia where hunting of small rodents and wild boars took place

and left overnight in a refrigerator or at room temperature. The dilution of such blood suspensions is approximately 1 : 25. Each such blood suspension was preliminary tested for the presence of antibodies to leptospires with the addition of an antigen in the dilution of the sera of 1 : 50, while each serum of the swine was preliminary examined in the initial dilution of 1 : 100. As antigens for testing the sera different serovars (sv.) of leptospires were used:
sv. *icterohaemorrhagiae* – RGA
sv. *ballum* – Mus 127
sv. *australis* – Ballico
sv. *pomona* – Pomona
sv. *grippotyphosa* – Moskow V
sv. *sejroe* – M84
sv. *saxkoebing* – M24
sv. *tarassovi* – Perepelicin
sv. *canicola* – Hond Utrecht IV
sv. *bataviae* – Van Tienen
sv. *hardjo* – Hardjoprajitno

The sera in which antibodies were established with one or more leptospira antigens in the initial dilution of 1 : 50 (rodents) or 1 : 100 (wild swine) were considered positive. After that each positive serum was titred with each of those antigens in a dilution from the initial one to the final titre.

Leptospira isolation

Tissue samples. The kidneys of 379 rodents and 154 wild boars were bacteriologically examined. After the dissection of rodents and swine, a small particle of kidney tissue the surface of which had previously been sterilized on flame, was inoculated in 5 test-tubes that contained 5 ml of Korthof's liquid medium (Johnson and Harris, 1967). The tubes with the inoculated material were incubated at 28° to 30°C and controlled each 7–10 days for the growth of leptospires during 35 to 45 days. The isolates were grown in 5 ml of Korthof's liquid medium prior to EMJH liquid medium at 30°C to get a density suitable for use in agglutination reactions with 23 standard antisera (group sera) for the first typing according to serogroup affinities (Babudieri, 1961; Ellinghausen and McCullough, 1965; Dikken and Kmety, 1978).

RESULTS

Results of rodent catching

In total 379 small rodents were caught, out of which 243 (64.1%) were females and 136 (35.9%) males. The species of small rodents were the following: *Apodemus (A.) agrarius*, *A. flavicolis*, *A. sylvaticus*, *Arvicola (A.) terrestis*, *Clethrionomys (C.) glareolus*, *Microtus (M.) agrestis* and *Mus musculus*. The prevailing species were *A. flavicolis* (111 animals) and *A. agraris* (108 animals), while the most numerous species of voles was *C. glareolus* (53 animals). According to the localities it was established that the largest number of animals was caught on the locality of Kutina (139 animals or 36.7% of the whole catch) (Table 1).

Results of serological analyses of rodent blood samples

The antibodies to leptospires were established in 48 (12.7%) out of 379 analysed blood samples of

Table 1. Number of caught rodents according to species and particular localities

Species	V. Gorica	Sisak	Kutina	N.Gradiska	Vrbanja	Gunja	Otok	Total	%
Apodemus agrarius	2	5	33	53	9	1	5	108	28.5
Apodemus flavicolis	10	24	46	17	6	2	6	111	29.3
Apodemus sylvaticus	9	13	13	7	7	7	6	62	16.4
Arvicola terrestis	0	0	1	0	0	0	0	1	0.3
Clethrionomys glareolus	5	3	13	21	4	6	1	53	13.9
Microtus agrestis	2	2	2	0	0	0	0	6	1.6
Microtus arvalis	3	0	0	1	1	0	1	6	1.6
Mus musculus	0	0	31	1	0	0	0	32	8.4
Total	31	47	139	100	27	16	19	379	100
%	8.2	12.4	36.7	26.4	7.1	4.2	5.0	100	

rodents from all of the localities investigated. Most frequently the antibodies were established in the following species: *Mus musculus* (34.4%), *A. agrarius* (14.8%), *A. flavicolis* (10.8%), *C. glareolus* (9.4%) and *A. sylvaticus* (6.5%). The antibodies were not established in the species *M. agrestis*, *M. arvalis* and *A. terrestis* (Table 2.).

In the blood sera of rodents the antibodies to antigens of nine different leptospira serovars were established. The antibodies to the following serovars were found most frequently: sv. *pomona* in 13 (27.1%) blood samples of rodents, sv. *sejroe* in 10 (20.8%) samples, sv. *australis* in 7 (14.6%), sv. *hardjo* in 6 (12.5%), sv. *saxkoebing* in 4 (8.3%), sv. *tarassovi* in 3 (6.3%), sv. *grippotyphosa* and sv. *bataviae* in 2 (4.2%) and sv. *icterohaemorrhagiae* in 1 (2.1%) blood sample (Table 3). The antibody titres for positive sera of rodents varied from 1 : 100 to 1 : 6 400. Most frequently the reactions were established in the serum dilution of 1 : 100 (41.6%) and 1 : 200 (25%), while in the highest dilution (1 : 6 400) the reactions were established in 4.2% of the sera of rodents (Table 4).

Results of serological analyses of blood samples of wild boars

In total the blood samples of 154 wild boars were analysed, out of which 85 (55.2%) were female and 69 (44.8%) male animals. The animals were between 4 months and 3 years of age, weighing between 24 and 110 kilograms. Positive reactions were established in 40 (26%) out of 154 examined blood samples of wild boars. Most positive reactions in wild boars were established in the region

Table 2. Findings of antibodies to leptospires in particular rodent species

Species	Examined	Positives	%
Apodemus agrarius	108	16	14.8
Apodemus flavicolis	111	12	10.8
Apodemus sylvaticus	62	4	6.5
Clethrionomys glareolus	53	5	9.4
Microtus agrestis	6	0	0
Microtus arvalis	6	0	0
Arvicola terrestis	1	0	0
Mus musculus	32	11	34.4
Total	379	48	12.7

Table 3. Findings of antibodies of different leptospira serovars in particular rodent species

Species	+/%	sv.aust.	sv.pom.	sv.ictero.	sv.gripp.	sv.taras.	sv.bat.	sv.sej.	sv.sax.	sv.har.
Mus musculus	11/22.9	0	0	0	0	0	0	7	2	2
Apodemus agrarius	16/33.3	0	7	1	2	2	0	1	0	3
Apodemus flavicolis	12/25.0	6	2	0	0	0	2	0	2	0
Clethrionomys glareolus	5/10.4	1	2	0	0	0	0	1	0	1
Apodemus sylvaticus	4/8.4	0	2	0	0	1	0	1	0	0
Total/%	48/100	7/14.6	13/27.1	1/2.1	2/4.2	3/6.2	2/4.2	10/20.8	4/8.3	6/12.5

Serovars:
sv.aust. – *australis*, sv.pom. – *pomona*, sv.ictero. – *icterohaemorrhagiae*, sv.gripp. – *grippotyphosa*, sv.taras. –- *tarassovi*, sv.bat. – *bataviae*, sv.sej. – *sejroe*, sv.sax. – *saxkoebing*, sv.har. – *hardjo*

Table 4. Titres of antibodies of different leptospira serovars found in small rodents

Sv. leptospires	Titres							
	+	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1 600	1 : 3 200	1 : 6 400
Sv. *australis*	7	3	2	0	1	0	1	0
Sv. *pomona*	13	5	2	3	0	1	1	1
Sv. *icterohaemorrhagiae*	1	0	1	0	0	0	0	0
Sv. *grippotyphosa*	2	1	0	0	1	0	0	0
Sv. *tarassovi*	3	1	1	0	1	0	0	0
Sv. *bataviae*	2	1	1	0	0	0	0	0
Sv. *sejroe*	10	6	2	0	0	1	0	1
Sv. *saxkoebing*	4	1	1	1	0	1	0	0
Sv. *hardjo*	6	2	2	1	1	0	0	0
Total/%	48/100	20/41.6	12/25.0	5/10.4	4/8.3	3/6.3	2/4.2	2/4.2

Table 5. Number of examined and positive wild boars in different regions

Regions	Examined	Positive	%
Velika Gorica	52	11	21.1
Sisak	17	5	29.4
Kutina	2	1	50.0
Nova Gradiska	21	9	42.9
Vrbanja	19	4	21.0
Gunja	19	4	21.0
Otok	24	6	25.0
Total	154	40	26.0

of Nova Gradiska (42.9%), and in other regions positive reactions were established in 21% to 29.4% of wild boars (Table 5). In wild boars sv. *pomona* was most frequently established i.e. in 19 (47.5%) animals, then sv. *australis* in 16 (40%), sv. *grippotyphosa* in 4 (10%) and sv. *icterohaemorrhagiae* in 1 (2.5%) animal. The antibody titre of wild swine reacting positively varied from 1 : 100 to 1 : 6 400. Most frequently the reactions were established in the titre of 1 : 100 and 1 : 200 (75%). A high titre of antibodies (1 : 6 400) was established in boars and sows (1 : 3 200) (Table 6). In Figure 2 the incidence of particular serovars of leptospires in small rodents and wild swine is presented. In small rodents and wild swine a high rate of coincidence for sv. *pomona* and sv. *australis* was established and at lower rate also for sv. *grippotyphosa* and sv. *icterohaemorhagiae* on common localities.

Results of bacteriological analysis

By the method of renoculture 17 (4.5%) isolates of leptospires were obtained from small rodents caught on the localities of Velika Gorica, Kutina and Nova Gradiska. The isolates were obtained from the following species: *Mus musculus* 10 (31.3%) isolates out of 32 rodents analysed; *A. agrarius* 4 (2.7%) isolates out of 108 rodents analysed and *A. flavicolis* 3 (2.7%) isolates out of 111 rodents analysed.

Serological analyses of 17 isolates of *Leptospira spp.* showed that the isolates identified belonged to three

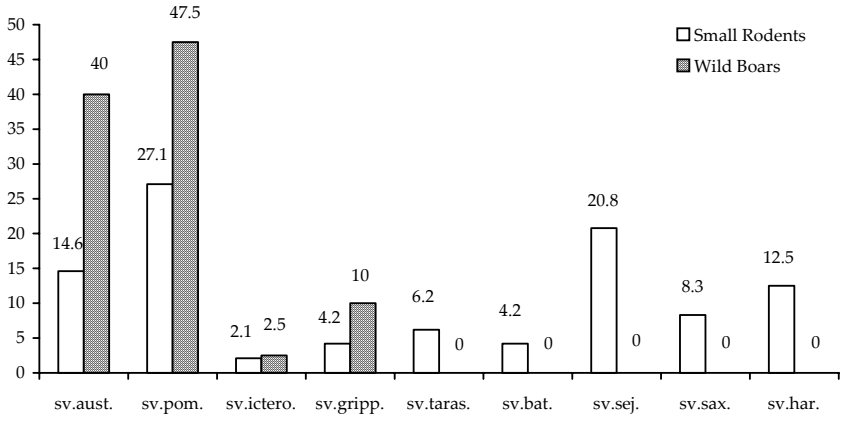

Figure 2. Incidence of different serovars of leptospires in small rodents and wild swine

sv.aust. – *australis*, sv.pom. – *pomona*, sv.ictero. – *icterohaemorrhagiae*, sv.gripp. – *grippotyphosa*, sv.taras. –- *tarassovi*, sv.bat. – *bataviae*, sv.sej. – *sejroe*, sv.sax. – *saxkoebing*, sv.har. – *hardjo*

Table 6. Serovars of leptospires and positive titres found in wild boars by serological analyses

Sv. leptospires	Titres							
	+/%	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1 600	1 : 3 200	1 : 6 400
Sv. *icterohaemorrhagiae*	1/2.5	1	0	0	0	0	0	0
Sv. *pomona*	19/47.5	8	5	1	3	0	1	1
Sv. *grippotyphosa*	4/10.0	3	0	0	0	1	0	0
Sv. *australis*	16/40.0	7	6	1	1	1	0	0
Total/%	40/100	19/47.5	11/27.5	2/5.0	4/10.0	2/5.0	1/2.5	1/2.5

serological groups: sv. *sejroe* (10 isolates), sv. *pomona* (5 isolates) and sv. *australis* (1 isolate), while one isolate was not identified. Ten isolates of sv. *sejroe* were obtained from the species *Mus musculus*, five isolates of sv. *pomona* were identified (4 from the species *A. agrarius* and 1 from *A. flavicolis*) and one isolate of sv. *australis* (*A. flavicolis*), while one isolate from *A. flavicolis* was not determined.

Out of 154 samples analysed by renoculture 13 (8.4%) isolates were obtained from the kidneys of wild boars. The samples originated from the folowing localities: Velika Gorica (5), Nova Gradiska (5), Otok (2) and Sisak (1). The isolates belonged to the following serological groups: *pomona* (10 isolates), *australis* (2 isolates) and *icterohaemorrhagiae* (1 isolate). The obtained isolates of leptospires in rodents and wild swine coincided with serological results.

DISCUSSION

The epizooties of leptospirosis can be understood only if they are observed as a broader biological phenomenon, as is the case with many other anthropozoonoses. The age-long persistence of *Leptospira* genus is enabled by animal life. On the list of leptospira reservoirs there is a large number of animals, mostly vertebrate-mammals, birds and amphibians. These investigations were mostly carried out in flooded and swampy regions of Croatia along the streamflow of the Sava river, in woody regions where the prevalence of common oak (*Quercus robur* L.) was 77%. Small rodents belong to a group of biotic factors which affect the quality of natural and artificial renewal of woods and forests. They regularly live in forests where they often cause damages on forest seeds and young plants. Small rodents make damage in all forests and in Croatia the greatest damages were registered in lowland forests of common oaks (Margaletic,1998). Most often these are the rodents from a subfamily *Murinae* (mice) and *Arvicolinae* (voles) (Wilson and Reeder, 1992). The term "small rodents" is used for the species from the order *Rodentia* (rodents) in which the body mass of adult animals is higher than 2 grams and less than 120 grams (Delany, 1974).

Small rodents are also the source of leptospires in nature. The persistence of leptospires in nature is enabled by a so-called "basic host" which in particular natural biocenosis and symbiosis makes their

persistence possible. This is known for sv. *grippotyphosa* where the basic host is *Microtus arvalis*, for sv. *pomona* and *Apodemus agrarius* and swine, sv. *sejroe* and *Mus musculus*, sv. *saxkoebing* and *Apodemus flavicolis* (Borcic *et al*., 1982).

In our study, the average findings of small rodents serologically positive to leptospirosis showed to be rather high, especially in the species *Mus musculus.* Furthermore, apart from *Mus musculus,* animals belonging to the species *A. agrarius, A. flavicollis, C. glareolus* and *A. sylvaticus* have shown to be the prevailing species infected by leptospires in Croatia. Borcic *et al.* (1978) indicated a striped field mouse (*A. agrarius*) and a forest vole (*C. glareolus*), and Treml *et al.* (2002) the species from the genus *Apodemus* sp. and *Microtus arvalis* as the prevailing species infected by leptospiras. Treml *et al.* (2002) found this species in the Czech Republic and established positive reactions to leptospirosis for sv. *grippotyphosa* in 20.6% of analysed animals. Similar findings in voles were described in the Netherlands by Kuiken *et al.* (1991). Stanko *et al.* (1996) found the antibodies to leptospires in 5% of small rodents (*A. flavicolis, A. agrarius* and *C. glareolus)* analysed in the eastern part of Slovakia, and sv. *grippotyphosa* was the prevailing serovar found. Adler *et al.* (2002) described the isolation of leptospires in Switzerland in 12.6% of rodents caught in the urban part of Zurich. Collares-Pereira *et al.* (2000) described the findings of leptospires in *Mus domesticus* in Portugal. Bondarenko *et al.* (2002) found sv. *grippotyphosa* and sv. *sejroe* in wild rodents from the Kirov region in Russsia. In the U.S.A. Songer *et al.* (1983) analysed 358 rodents from 6 localities in Arizona and isolated leptospires in 10.4% of samples and identified sv. *ballum* as a prevailing serovar. Cho *et al.* (1998) described the findings of leptospirosis in 9.9% of rodents of the species *Apodemus agrarius* in Korea, and isolated *L. icterohaemorhagiae* sv. *lai*.

From 379 samples of rodent kidneys bacteriologically analysed 17 (4.5%) isolates of leptospires were isolated (*Mus musculus* – 10 isolates, *A. agrarius* – 4 isolates and *A. flavicolis* – 3 isolates). The isolates were classified into three serological groups: *sejroe* (10), *pomona* (5) and *australis* (1), and one isolate was not determined. By the analysis of macrorestriction of chromosomal DNA "PFGE" Turk *et al.* (2003) demonstrated that ten isolates identified from *Mus musculus* showed the greatest resemblance to sv. *istrica* of *sejroe* serological group, genomic species *L. borgpetersenii*. Five isolates (4 isolates from *A. agrarius* and 1 from *A. flavicolis*) belonged to sv. *tsaratsovo* of *pomona* serological group, genomic species *L. kirshneri.* One isolate (isolated from *A. flavicolis*) belonged to sv. *lora* of *australis* serological group, genomic species *L. interrrogans*. One isolate obtained from *A. flavicolis* was not determined.

In wild boars positive serological reactions were established in 26% of analysed blood samples. In total 13 (8.4%) isolates were obtained which belonged to the following serological groups: *pomona* (10 isolates), *australis* (2 isolates) and *icterohaemorhagiae* (1 isolate). In Croatia there are only few studies considering the prevalence of leptospirosis in wild boars. Kovacic *et al.* (1984) reported on the results of investigations carried out in the region of Baranja where 9.2% of wild boars showed to be positive and the most frequent serovars were: sv. *pomona*, sv. *grippotyphosa*, *australis* and *tarrasovi*. Borcic *et al.* (1989) investigated the presence of leptospirosis in red deer, roe-deer, hares and wild boars. The authors reported that the antibodies were determined mostly in wild boars. On nine localities included in the investigation the antibodies to leptospires were established in averagely 28.1% of wild boars. Kovacic *et al.* (2001) reported on the findings of positive reactions in 6.2% of wild boars in the region of Gorski Kotar. Cvetnic *et al.* (2002) in their investigation in the region of Turopolje established the prevalence of identical serovars of leptospires (sv. *australis* and sv. *pomona*) in rodents and in Turopolje swine from the same region. Similar findings were described by Mason *et al.* (1998), Saliki *et al.* (1998), Vicente *et al.* (2002), Treml *et. al.* (2003).

The high percentages of antibodies to sv. *pomona* and sv. *australis* found in small rodents and wild boars on common localities indicate that there is a possibility of contact between wild boars and rodents and the spreading of the infection between them. This is certainly favoured by the environment, wet and swampy soil that provides conditions for the growth of leptospires and exactly in such regions these investigations were carried out. Morales *et al.* (1978) described the isolation of sv. *pomona* leptospires from the kidneys of rats which were the source of sv. *pomona* on a swine farm. Whyte and Ratcliff (1982) incriminated a field mouse (*Apodemus agrarius*) as a source of leptospirosis sv. *pomona* on a swine-breeding farm. Kuiken *et al.* (1991) indicated voles (*Microtus arvalis*) as possible sources of leptospires sv. *hardjo* and sv. *grippotyphosa* also in cattle.

The results of our investigations confirmed the presence of *L. interrogans* sv. *pomona, saxkoebing, hardjo, australis, tarassovi, sejroe, gripotyphosa, bataviae*

and *icterohaemorhagiae* in small rodents and the presence of sv. *pomona*, *australis*, *icterohaemorrhagiae* and *grippotyphosa* in wild boars from several localities in Croatia. On the basis of our study it can be concluded that small rodents and wild boars are natural reservoirs of leptospires in particular regions of Croatia and represent a significant potential source of leptospirosis for other wild and domestic animals as well as humans.

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