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 \textit{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 \textit{et al.}, 1978). Borcic \textit{et al.} (1982, 1983) determined rodents as reservoirs of leptospires in the valleys of the rivers Sava and Drava.

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 terrofauna 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.

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.
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. tarassovi* – M24
- *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...
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

<table>
<thead>
<tr>
<th>Species</th>
<th>Examined</th>
<th>Positives</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Apodemus agrarius</em></td>
<td>108</td>
<td>16</td>
<td>14.8</td>
</tr>
<tr>
<td><em>Apodemus flavicolis</em></td>
<td>111</td>
<td>12</td>
<td>10.8</td>
</tr>
<tr>
<td><em>Apodemus sylvaticus</em></td>
<td>62</td>
<td>4</td>
<td>6.5</td>
</tr>
<tr>
<td><em>Clethrionomys glareolus</em></td>
<td>53</td>
<td>5</td>
<td>9.4</td>
</tr>
<tr>
<td><em>Microtus agrestis</em></td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Microtus arvalis</em></td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Arvicola terrestis</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>32</td>
<td>11</td>
<td>34.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>379</td>
<td>48</td>
<td>12.7</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td><em>Mus musculus</em></td>
<td>11/22.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>Apodemus agrarius</em></td>
<td>16/33.3</td>
<td>0</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><em>Apodemus flavicolis</em></td>
<td>12/25.0</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Clethrionomys glareolus</em></td>
<td>5/10.4</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><em>Apodemus sylvaticus</em></td>
<td>4/8.4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Total/%</strong></td>
<td>48/100</td>
<td>7/14.6</td>
<td>13/27.1</td>
<td>1/2.1</td>
<td>2/4.2</td>
<td>3/6.2</td>
<td>2/4.2</td>
<td>10/20.8</td>
<td>4/8.3</td>
<td>6/12.5</td>
</tr>
</tbody>
</table>

Serovars:

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

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

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

<table>
<thead>
<tr>
<th>Regions</th>
<th>Examined</th>
<th>Positive</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Velika Gorica</td>
<td>52</td>
<td>11</td>
<td>21.1</td>
</tr>
<tr>
<td>Sisak</td>
<td>17</td>
<td>5</td>
<td>29.4</td>
</tr>
<tr>
<td>Kutina</td>
<td>2</td>
<td>1</td>
<td>50.0</td>
</tr>
<tr>
<td>Nova Gradiska</td>
<td>21</td>
<td>9</td>
<td>42.9</td>
</tr>
<tr>
<td>Vrbanja</td>
<td>19</td>
<td>4</td>
<td>21.0</td>
</tr>
<tr>
<td>Gunja</td>
<td>19</td>
<td>4</td>
<td>21.0</td>
</tr>
<tr>
<td>Otok</td>
<td>24</td>
<td>6</td>
<td>25.0</td>
</tr>
<tr>
<td>Total</td>
<td>154</td>
<td>40</td>
<td>26.0</td>
</tr>
</tbody>
</table>

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. icterohaemorrhagiae 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
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. flavidus) and one isolate of sv. australis (A. flavidus), while one isolate from A. flavidus 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 following 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 anthrozoanoses. 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 (Boricic 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. Boricic et al. (1978) indicated a striped field mouse (A. agrarius) and a forest vole (C. glareolus), and Tremel et al. (2002) the species from the genus Apodemus sp. and Microtus arvalis as the prevailing species infected by leptospirosis. Tremel 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 leptospirosis in 5% of small rodents (A. flavicollis, 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 leptospirosis in Mus domesticus in Portugal. Bondarenko et al. (2002) found sv. grippotyphosa and sv. sejroe in wild rodents from the Kirov region in Russia. 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. flavicollis – 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. ictrica of sejroe serological group, genomic species L. borgpetersenii. Five isolates (4 isolates from A. agrarius and 1 from A. flavicollis) belonged to sv. tsaratsovo of pomona serological group, genomic species L. kirshneri. One isolate (isolated from A. flavicollis) belonged to sv. lora of australis serological group, genomic species L. interrogans. One isolate obtained from A. flavicollis 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 tarassovi. Boricic 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), Tremel 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. gippotyphosa also in cattle.

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

REFERENCES


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