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Epizootiological survey of small mammals as *Leptospira* spp. reservoirs in Eastern Croatia

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Abstract

In this survey we investigated a population of small mammals in Eastern Croatia in order to determine *Leptospira* carriage rates and identify circulating serovars. Out of 67 trapped animals, 20 (29.9%) isolates were obtained. Identification of isolates using microscopic agglutination test, pulsed field gel electrophoresis and multi locus sequence typing revealed that 10 (50.0%) isolates belong to serogroup Pomona, serovar Mozdok, 6 (30.0%) isolates to serogroup Australis, serovar Jalna, 2 (10.0%) isolates to serogroup Sejroe, serovar Saxkoebing, and 1 (5.0%) isolate to serogroup Grippotyphosa, serovar Grippotyphosa. One isolate from serogroup Bataviae was unable to be identified to the serovar level. Amplification of a 331-bp region of the locus LA0322 using real-time polymerase chain reaction determined that 12 (60.0%) isolates belong to *L. kirschneri,* 6 (30.0%) isolates to *L. interrogans,* and 2 (10.0%) isolates to *L. borgpetersenii. Leptospira* carriage rate was high (29.9%), which corresponds to a high incidence of human and domestic animal leptospirosis in Eastern Croatia. Furthermore, 90.0% of the isolates belong to serogroups Pomona, Australis and Sejroe which are also the most prevalent serogroups in humans in this area. These findings suggest that small mammals might be an important source of *Leptospira* spp. infection in Eastern Croatia.

Keywords

Leptospira; Leptospirosis; Reservoirs; Small mammals; Molecular; Zoonosis

1. Introduction

Leptospirosis is a zoonosis of worldwide distribution, caused by pathogenic spirochetes of the genus *Leptospira* (Levett, 2001). It affects all mammals, including humans, livestock and

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wildlife. Leptospires are very heterogenous and are classified phenotypically and genetically. Phenotypic classification with the serovar as the basic taxon is based on antigenic differences determined by the cross agglutination-absorption test (CAAT). So far, more than 300 serovars have been identified and grouped into 29 serogroups. Genetic classification is based on DNA homology and divides the genus *Leptospira* into 20 species (Smythe et al., 2013). Due to very demanding requirements and difficult implementation of CAAT, in the last few decades various molecular methods for identification of isolates to the serovar level have been developed. For that purpose pulsed field gel electrophoresis (Galloway and Levett, 2008, 2010), restriction fragment length polymorphism-based methods (Perolat et al., 1994), arbitrarily primed polymerase chain reaction (Ralph et al., 1993), variable number of tandem repeat analysis (Majed et al., 2005; Slack et al., 2005) and multi locus sequence typing (Thaipadungpanit et al., 2007) have been used. Genomic species of *Leptospira* spp. can be determined with various molecular methods, mostly based on 16S rDNA analysis. In the last decade, efforts have been made to develop real-time PCR for detection of pathogenic *Leptospira* from cultures and clinical materials (Levett et al., 2005; Merien et al., 2005; Fearnley et al., 2007; Ahmed et al., 2009). However, the combination of both molecular and serological identification methods still provides the best results in most cases.

Identification of *Leptospira* spp. isolates from small mammals is required to make a connection between human and animal disease and small mammal reservoirs because most infections are acquired from an environment contaminated with infected urine. Since clinical manifestations and disease outcome, among other things, might depend on the infecting *Leptospira* serovar, it is also of interest for clinicians to know what the circulating serovars in particular areas are.

Surveys conducted in the past decades revealed that Croatia is an endemic area of leptospirosis, especially in valleys of big rivers that flow from west to east through most of the lowland (Borcic et al., 1982; Milas et al., 2002; Turk et al., 2003). The importance of leptospirosis in Croatia is also highlighted by the official data from the Croatian National Institute of Health, according to which the mean yearly incidence of human leptospirosis from 1990 to 2007 was 1.83/100,000 inhabitants, with an incidence >2.5/100,000 inhabitants recorded approximately every 3–4 years (Balen Topic et al., 2010). These data make Croatia one of the countries with the highest incidence of human leptospirosis in Europe and in the world as well (Pappas et al., 2008). In the majority of previous reservoir surveys in Croatia, *Leptospira* carriage rates were investigated by serology and renal culture. Unfortunately, more thorough serological or molecular identification methods were unavailable so obtained isolates were not identified to the serovar level. Only in the latest study (Turk et al., 2003) were isolates identified to the serovar level, which set the ground for further epizootiological surveys.

The aim of this study was to determine *Leptospira* spp. carriage rates in a small mammal population and identify circulating serovars in order to correlate these data with incidence of human leptospirosis in Eastern Croatia.

2. Materials and methods

2.1. Animal trapping, sampling of kidneys and kidney culturing

During the relative abundance estimation of rodents in October 2005, at three sites in Eastern Croatia (Mikanovci, Cerna and Ilok), animals were trapped using 238 common snap traps positioned along transect lines (Fig. 1).

Species of animals was determined based on morphological characteristics. Animals were aseptically dissected. Kidney tissue was immediately inoculated into the homemade Korthof's medium and sampled for DNA extraction. Positive cultures were subcultured in Korthof's medium until they reached stable growth, then subcultured to Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium and cultivated up to a density of 2–4 × 10^8 leptospires per millilitre, suitable for serological and molecular identification procedures.

2.2. Microscopic agglutination test (MAT) with rabbit antisera

To identify the obtained isolates to the serogroup level, the microscopic agglutination test (MAT) was performed following the standard procedure using a panel of 18 rabbit anti-*Leptospira* reference antisera. Reference rabbit antisera used in this study were from the Koninklijk Instituut voor de Tropen (KIT), Amsterdam, The Netherlands (Table 1).

2.3. DNA extraction from Leptospira cultures and animal kidneys

Reference strains and isolates were grown at 30°C in EMJH medium and harvested by centrifugation during the late logarithmic phase. Genomic DNA from animal kidneys and *Leptospira* isolates was extracted using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and stored at −20 °C.

2.4. Polymerase chain reaction (PCR)

Polymerase chain reaction was performed on all obtained isolates and kidneys of related animals using three primer pair sets. PCR with primers LeptoA (5′-GGCGGCGCGTCTTAAACATG-3′) and LeptoB (5′-TTCCCCCCATTGAGCAAGATT-3′) as described by Merien et al. (1992) was used to confirm the presence of *Leptospira* DNA in cultures and kidney tissue. Semi-nested PCR with two primer sets, L3 (5′-TGAGGGTTAAAACCCCCAAC-3′) and L4 (5′-GATTTTTCGGGTAAAGATT-3′) followed by L4 and Lepat2 (5′-TCACAT(CT)GCTGCTTATTTT-3′) as described by Gravekamp et al. (1993) was performed to confirm pathogenicity of *Leptospira* DNA present in cultures and kidney tissue. All products were electrophoresed in 1% agarose gel and compared to a molecular size marker.

2.5. Pulsed-field gel electrophoresis (PFGE)

Subsequent typing of the isolates to the serovar level was performed by pulsed-field gel electrophoresis (PFGE). Preparation of agarose plugs was performed as described by Galloway and Levett (2008). Genomic DNA of isolates from serogroups Pomona, Saxkoebing, Bataviae and Grippotyphosa was restricted with the endonuclease *NotI* and subjected to PFGE for 18 h with circulating 0.5 × TBE buffer. Electrophoresis conditions

were as follows: switch times of 2.16 and 35.07 s, angle of 120 °, gradient of 6 V/cm, temperature of 14 °C, and linear ramping factor. Genomic DNA of isolates from serogroup Australis was restricted with endonuclease *SgrAI* and subjected to PFGE for 22 h with circulating 0.5 × TBE buffer. Electrophoresis conditions were as follows: switch times of 5 and 30 s, angle of 120°C, gradient of 6 V/cm, temperature of 14°C, and linear ramping factor. Gels were stained with ethidium bromide and analysed with Gel Doc 2000 System (Bio-Rad Laboratories, Richmond, CA, USA).

2.6. Multi locus sequence typing (MLST)

Subsequent typing of the isolates from serogroup Australis to the serovar level was performed by multi locus sequence typing (MLST). MLST was performed by amplifying and sequencing seven housekeeping genes as previously described (Thaipadungpanit et al., 2007). Sequence types (STs) were determined from the resulting allelic profiles of the seven sequenced genes and compared to an established Internet database to obtain serovar identification (http://www.mlst.net).

2.7. Real-time PCR with Tm determination

Real-time PCR was applied as described by Merien et al. (2005) with one primer set, LEB1-F (5′-CATTCATGTTTCGAATCATTTCAAA-3′) and LEB1-R (5′-GGCCCAAGTTCCTTCTAAAAG-3′), that amplifies a 331-bp of the locus LA0322 obtained from the complete genome sequence of *L. interrogans* serovar Lai. Melting temperature (Tm) of PCR product was used to distinguish particular *Leptospira* genomic species. Real-time PCR was performed on all *Leptospira* isolates and kidneys of *Leptospira* spp. positive animals to determine genomic species of *Leptospira*. All specimens were tested ten times and mean melting temperatures were calculated.

3. Results

3.1. Identification of trapped animals and isolation of Leptospira spp. from kidneys

The study included 67 animals, 30 (44.8%) from Ilok, 28 (41.8%) from Mikanovci, and 9 (13.4%) from Cerna. Out of 67 trapped animals, 29 (43.3%) were identified as *Apodemus agrarius*, 20 (29.8%) as *A.flavicollis*, 9 (13.4%) as *A. sylvaticus*, 6 (8.9%) as *S. araneus*, 1 (1.5%) as *Muscardinus avellanarius*, 1 (1.5%) as *Microtus arvalis* and 1 (1.5%) as *Myodes glareolus*. Kidney cultures from 20/67 (29.9%) animals were positive for *Leptospira* spp.: 10/20 from *A. agrarius* (M613, M615, M616, M619, M621, M634, M635, M639, M640, M656), 5/20 from *A. flavicollis* (M664, M666, M675, M676, M678), 3/20 from *A. sylvaticus* (M641, M644, M649), 1/20 from *M. avellanarius* (M628) and 1/20 from *M. arvalis*. Results are shown in Table 2.

3.2. Microscopic agglutination test (MAT) with rabbit antisera

Serogroup identification of 20 isolates with 18 rabbit antisera revealed that 10/20 isolates belong to serogroup Pomona (M613, M615, M616, M619, M621, M628, M634, M635, M639, M656), 6/20 to serogroup Australis (M641, M644, M649, M666, M675, M678), 2/20 isolates to serogroup Sejroe (M664, M676), 1/20 isolate to serogroup Bataviae (M640) and 1/20 to serogroup Grippotyphosa (M631).

3.3. Pulsed-field gel electrophoresis and multi locus sequence typing

Patterns of ten isolates from serogroup Pomona (M613, M615, M616, M619, M621, M628, M634, M635, M639, M656) coincided with electrophoretic profile of reference strain *L. kirschneri* serovar Mozdok.

Patterns of two isolates from serogroup Saxkoebing (M664, M676) coincided with electrophoretic profile of reference strain *L. borgpetersenii* serovar Saxkoebing.

The pattern of the isolate from serogroup Grippotyphosa (M631) coincided with electrophoretic profile of reference strain *L. kirschneri* serovar Grippotyphosa.

The pattern of the isolate from serogroup Bataviae (M640) did not coincide with electrophoretic profile of any of the reference strains from *L. kirschneri* genomic species from serogroup Bataviae and remained undetermined. Selected PFGE patterns are shown in Fig. 2.

Patterns of six isolates from serogroup Australis (M641, M644, M649, M666, M675, M678) coincided with electrophoretic profiles of reference strains *L. interrogans* serovar Jalna and *L. interrogans* serovar Lora. Therefore, isolates from serogroup Australis were further analysed with MLST and identified as sequence type (ST) 24, which is consistent with *L. interrogans* serovar Jalna but different from *L. interrogans* serovar Lora (ST 25).

3.4. Polymerase chain reaction (PCR)

PCRs with three primer pairs (LeptoA–LeptoB, L3–L4 and L4–Lepat2), performed on DNAs extracted from all obtained *Leptospira* isolates and kidneys of animals from which *Leptospira* spp. were isolated, resulted in products with the expected size of 331 bp, 643 bp and 270 bp, respectively.

3.5. Real-time PCR with Tm determination

Out of 20 isolates, 12 had mean melting temperatures characteristic for *L. kirschneri* (M613, M615, M616, M619, M621, M628, M631, M634, M635, M639, M640, M656), 6 for *L. interrogans* (M641, M644, M649, M666, M675, M678) and two for *L. borgpetersenii* (M664 and M676). Results of real-time PCR performed on 20 kidneys of animals from which *Leptospira* spp. were isolated, coincided with results of real-time PCR performed on isolated *Leptospira* spp. cultures. Results of genomic species determination are shown in Table 3.

Results of serogroup, serovar and genomic species determination as well as methods used are summarised in Table 4.

4. Discussion

In this survey we investigated distribution and abundance of a mouse-like rodent population and their *Leptospira* carriage rates in Eastern Croatia. Moreover, we determined genetic and antigenic variability of circulating *Leptospira* spp. serovars.

Species identification of trapped animals and numbers of animals with positive renal cultures is presented in Table 2. Prevalence of *A. agrarius* can probably be explained by the fact that two of three investigated sites were small wooded areas surrounded by fields, a common habitat of this species. Increased abundance of *A. agrarius* in woods is also attributable to reduction of food sources in fields during autumn, when trapping was conducted. This is, to our knowledge, the first report of *Leptospira* spp. isolation from *M. avellanarius*. The carriage rate detected in this investigation (29.9%) and the carriage rate of 29.5% detected in another investigation conducted in Croatia by Tadin et al. (2012) are the highest reported carriage rates in small mammals. In Europe, carriage rate of 12.1% in Switzerland (Adler et al., 2002) was reported. From other parts of the world, carriage rates of 0% in Madagaskar (Ralaiarijaona et al., 2001), 10.5% in Japan (Koizumi et al., 2008), 11% in Tanzania (Mgode et al., 2005), 12.6% in Korea (Cho et al., 1998) and 15% in Thailand (Doungchawee et al., 2005) were reported. A higher carriage rate than in our study (82.9%) was reported in domestic mice in Terceira island (Collares-Pereira et al., 2000). The highest *Leptospira* carriage rates among small mammals are reported in rats worldwide (90.5% in USA by Vinetz et al., 1996, 27.1% in Turkey by Sunbul et al., 2001, 63% in Thailand by Niwetpathomwat and Doungchawee, 2005, 50% in India by Priya et al., 2007, 80% in Brazil by Faria et al., 2008). The high carriage rate detected in this investigation further shows that Croatia is an endemic area of leptospirosis, especially in valleys of big rivers Sava and Drava (Borcic et al., 1982; Milas et al., 2002).

Serogrouping of isolates revealed that 10/20 belong to serogroup Pomona, 6/20 to serogroup Australis, 2/20 to serogroup Sejroe, 1/20 to serogroup Bataviae and 1/20 to serogroup Grippotyphosa. These results correlate to the latest serological surveys on human leptospirosis in Eastern Croatia which reports Pomona, Australis and Sejroe as the most prevalent serogroups (Peric et al., 2005; Cvitkovic, 2007). In this study 9/10 (90.0%) of *L. kirschnerii* serovar Mozdok serogroup Pomona were isolated from *A. agrarius*, which confirms this species as natural host of *L. interrogans* serogroup Pomona (Borcic et al., 1986).

Discrepancies between humoral response and actual carriership confirmed by renal culture or PCR were reported by several authors (Sunbul et al., 2001; Mgode et al., 2005; Priya et al., 2007). Due to evolutionary adaptation of rodent reservoirs to *Leptospira* spp. infection, serology is very limited in determination of carrier status. Low or absent antibody production in response to infection results in poor correlation between humoral response and actual carriership in reservoirs. That means that serological testing of reservoir animals certainly cannot be used for investigation of carriage rates and especially not for predicting the leptospiral serovars responsible for the infection. Knowing that, it is obvious that data on *Leptospira* carriage rates in most parts of the world are very insufficient. It is also unreliable to determine the infecting serovar or even serogroup based on serological testing.

Even in humans, where a stronger correlation of humoral response and infecting serovar is expected, it is often not possible to infer the infecting *Leptospira* serovar from the results of serological testing, especially in the early stage of infection (Levett, 2003; Murray et al., 2011). Identification of isolates to the serovar level is very important for complete understanding of epizootiology and epidemiology of the disease. We used pulsed-field gel

electrophoresis (PFGE) and multi locus sequence typing (MLST) to identify 20 *Leptospira* isolates to the serovar level. Real time polymerase chain reaction (real-time PCR) with determination of melting temperature (Tm) was used to determine genomic species of *Leptospira* from cultures and kidney tissues.

PCR based Tm revealed that 12/20 (60.0%) isolates belong to *L. kirschneri* (10 to serovar Mozdok, one to serovar Grippotyphosa and one to undetermined serovar), 6/20 (30.0%) isolates to *L. interrogans* (serovar Jalna) and 2/20 (10.0%) isolates to *L. borgpetersenii* (serovar Saxkoebing).

Those results differ from a previously conducted study of small mammals in different parts of Croatia which reports 62.5% of isolates as *L. borgpetersenii* serovar Istrica, 31.3% as *L. kirschneri* serovar Tsaratsovo and 6.3% as *L. interrogans* serovar Lora (Turk et al., 2003). The variety of serovars observed in these two studies illustrates the overall diversity of *Leptospira* serovars present in Croatia.

Real-time PCR detected *Leptospira* spp. DNA in the kidneys of all animals from which *Leptospira* spp. isolates were obtained. At the same time, genomic species of *Leptospira* in tissue was determined. Results of genomic species determination from kidney tissue correlated with results obtained for *Leptospira* isolates cultures. The method we used is standardized for determination of *Leptospira* genomic species from cultures but is not widely used for determination and identification of *Leptospira* from tissues. Our investigation suggests that there is no interference of rodent DNA or any other limitations for using this method in reservoir surveys when culturing of *Leptospira* is not feasible due to either field or laboratory conditions as well as personnel skills. Due to the high carriage rate found, we can conclude that Croatia remains an endemic area of leptospirosis. Accordance of serogroups previously detected in humans and herein in animals indicates that small mammals might be a main source of infection for humans and animals. Analysis of isolated *Leptospira* to the serovar level revealed great genetic diversity of *Leptospira* circulating in Croatia.

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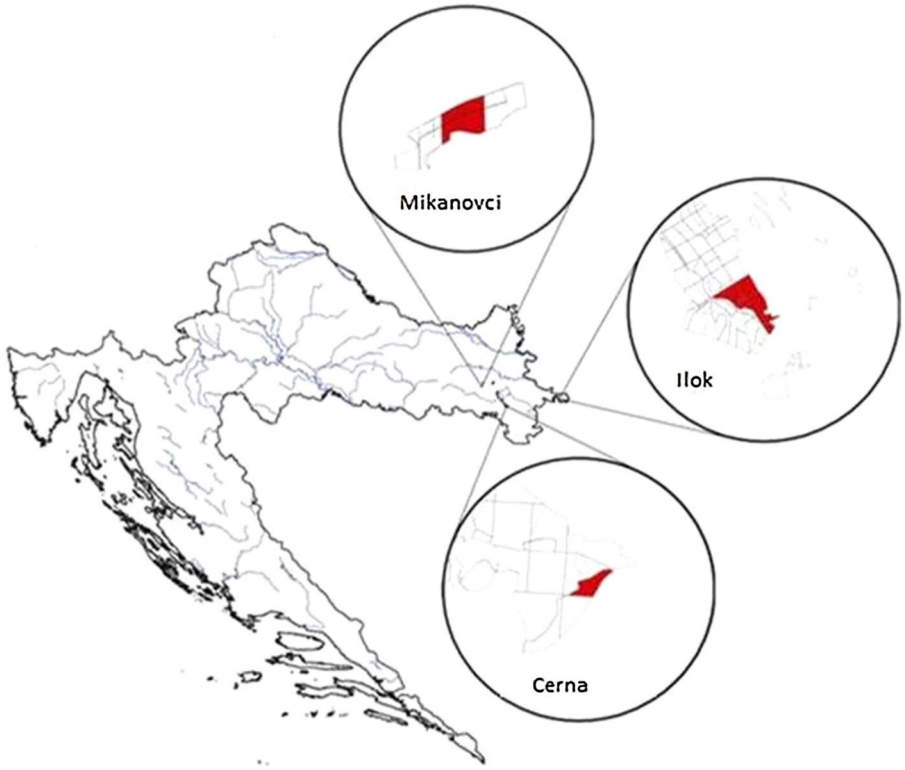

Fig. 1.
Localities in Eastern Croatia where animals were trapped.

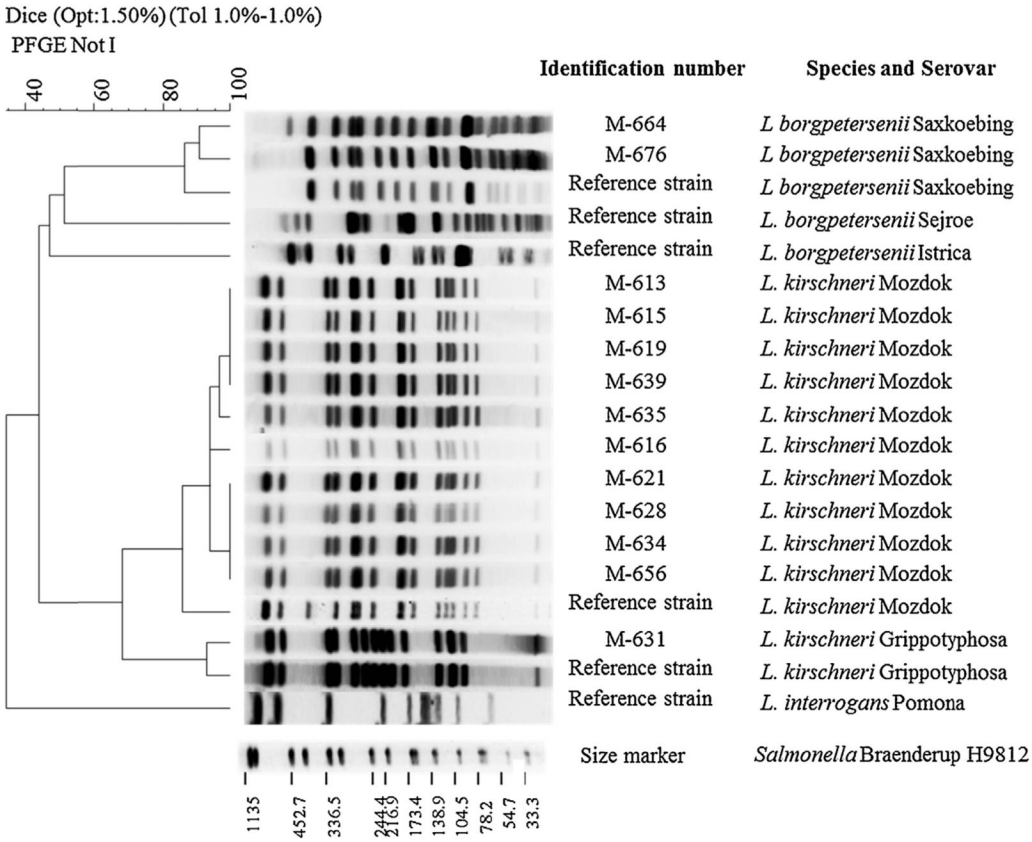

Fig. 2.

Dendrogram and electrophoretic profiles of *NotI*-restriction of whole genome of selected *Leptospira* isolates and reference strains.

Table 1

Reference strains used in production of rabbit antisera.

Serogroup	Serovar	Strain
Australis	Australis	Ballico
Autumnalis	Autumnalis	Akiyami A
Bataviae	Bataviae	Van Tienen
Ballum	Castellonis	Castellon 3
Canicola	Canicola	Hond Utrecht IV
Cynopteri	Cynopteri	3522C
Grippotyphosa	Grippotyphosa	Moskva V
Hebdomadis	Hebdomadis	Hebdomadis
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
Icterohaemorrhagiae	Copenhageni	M 20
Panama	Panama	CZ 214K
Pomona	Pomona	Pomona
Pyrogenes	Pyrogenes	Salinem
Semaranga	Patoc	Patoc I
Sejroe	Hardjo	Hardjoprajitno
Sejroe	Saxkoebing	Mus 24
Sejroe	Sejroe	M 84
Tarassovi	Tarassovi	Mitis Johnson

Table 2

Localities where animals were trapped, identified species of animals and numbers of animals with positive renal cultures.

Locality/species	*A. agrarius*	*A. flavicollis*	*A. sylvaticus*	*M. avellanarius*	*M. arvalis*	*S. araneus*	*M. glareolus*	Total
Mikanovci	9/18	0/2	0/0	1/1	1/1	0/6	0/0	11/28
Cerna	1/9	0/0	0/0	0/0	0/0	0/0	0/0	1/9
Ilok	0/2	5/18	3/9	0/0	0/0	0/0	0/1	8/30
Total	10/29	5/20	3/9	1/1	1/1	0/6	0/1	20/67

Table 3

PCR based determination of melting temperatures (Tm) fo.

Isolate	Tm range/mean	Determined genomic species
M 613	(84.31–84.76)/84.59	*L. kirschneri*
M 615	(84.32–84.74)/84.49	*L. kirschneri*
M 616	(84.23–84.84)/84.55	*L. kirschneri*
M 619	(84.21–84.73)/84.47	*L. kirschneri*
M 621	(84.12–84.70)/84.48	*L. kirschneri*
M 628	(84.12–84.69)/84.48	*L. kirschneri*
M631	(84.05–84.79)/84.53	*L. kirschneri*
M 634	(84.08–84.79)/84.22	*L. kirschneri*
M 635	(84.10–84.80)/84.55	*L. kirschneri*
M 639	(84.02–84.74)/84.41	*L. kirschneri*
M 640	(84.44–84.84)/84.62	*L. kirschneri*
M 641	(83.39–83.71)/83.60	*L. interrogans*
M 644	(83.39–83.70)/83.56	*L. interrogans*
M 649	(83.29–83.77)/83.60	*L. interrogans*
M 656	(84.22–84.72)/84.54	*L. kirschneri*
M 664	(86.00–86.51)/86.35	*L. borgpeterseni*
M 666	(83.25–83.69)/83.61	*L. interrogans*
M 675	(83.19–83.72)/83.61	*L. interrogans*
M 676	(85.94–86.30)/86.24	*L. borgpeterseni*
M 678	(83.18–83.63)/83.58	*L. interrogans*

Table 4

Results of identification and methods used.

Isolate	Serogroup determined by MAT	Serovar determined by PFGE	Serovar determined by MLST	Genomic species determined by real time PCR(Tm)
M 613	Pomona	Mozdok		*L. kirschneri*
M 615	Pomona	Mozdok		*L. kirschneri*
M 616	Pomona	Mozdok		*L. kirschneri*
M 619	Pomona	Mozdok		*L. kirschneri*
M 621	Pomona	Mozdok		*L. kirschneri*
M 628	Pomona	Mozdok		*L. kirschneri*
M631	Grippotyphosa	Grippotyphosa		*L. kirschneri*
M 634	Pomona	Mozdok		*L. kirschneri*
M 635	Pomona	Mozdok		*L. kirschneri*
M 639	Pomona	Mozdok		*L. kirschneri*
M 640	Bataviae	Undetermined	Undetermined	*L. kirschneri*
M 641	Australis	Undetermined	Jalna	*L. interrogans*
M 644	Australis	Undetermined	Jalna	*L. interrogans*
M 649	Australis	Undetermined	Jalna	*L. interrogans*
M 656	Pomona	Mozdok		*L. kirschneri*
M 664	Sejroe	Saxkoebing		*L. borgpetersenii*
M 666	Australis	Undetermined	Jalna	*L. interrogans*
M 675	Australis	Undetermined	Jalna	*L. interrogans*
M 676	Sejroe	Saxkoebing		*L. borgpetersenii*
M 678	Australis	Undetermined	Jalna	*L. interrogans*