Epidemiol. Infect. (2003), **130**, 159–166. © 2003 Cambridge University Press
DOI: 10.1017/S0950268802008026 Printed in the United Kingdom

Molecular characterization of *Leptospira* spp. strains isolated from small rodents in Croatia

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(*Accepted 2 October 2002*)

SUMMARY

We report the isolation and characterization of 16 *Leptospira* spp. strains isolated from small rodents captured in 11 different regions of inland Croatia. Large *Not*I and *Sgr*AI restriction fragment allowed us to assign 10 isolates to the serovar istrica, 5 isolates to the serovar tsaratsovo and 1 isolate to the serovar lora. The phylogenetic analysis conducted from the sequences of the first 330 bp from the 16S rDNA gene revealed that the strains belonged to three different species, *L. borgpetersenii*, *L. kirschneri* and *L. interrogans*. Carrier rates in eight rodent species varied from 0 to 71·4%. *Mus musculus* showed the highest infection level and confirmed its role as a major reservoir of the serogroup Sejroë. For the first time we reported the occurrence of serovars tsaratsovo and lora in Croatia.

INTRODUCTION

Leptospirosis is a zoonosis widespread throughout the world, caused by pathogenic members of the genus *Leptospira* with great impact on both human and veterinary public health. Leptospires are immunologically and genetically heterogeneous spiral-shaped microorganisms that comprise 223 serovars organized into 24 serogroups [1] and several species [2–5]. Although leptospirosis was first described in Croatia in 1935 [6], our understanding of the circulating leptospiral serovars in circulation is still scarce. Various small rodents serve unambiguously as reservoir hosts for leptospires and they have potential to shed them in urine for extended periods. The evidence that small rodents carry leptospires and the presence of antileptospiral agglutinins have been previously reported in certain parts of inland Croatia [7, 8]. To obtain a clear picture of the occurrence of the most common serovars, we started investigating the prevalence and identification of leptospira in small rodents in known natural foci of leptospirosis in Croatia.

There are various antigenic and genetic methods for the identification and characterization of leptospires. In the past years in Croatia new isolated serovars were originally classified on the basis of their antigenic traits following the conventional serotyping methods – the microscopic agglutination test and the cross-agglutinin absorption test (CAAT) with rabbit antisera [9]. Serovar identification by CAAT is fastidious, time-consuming, requires large volumes of culture and is hampered by its subjective interpretation (due to dark-field microscopy estimation of a 50% agglutinated leptospires cut-off). To overcome such difficulties,

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Table 1. *Strains used in the study*

Serogroup	Serovar	Strain (collection)*	Genomic species
Australis	lora	Lora (IP)	*L. interrogans s.s.*
Australis	jalna	Jalna (IP)	*L. interrogans s.s.*
Australis	jalna	Jalna (KIT)	*L. interrogans s.s.*
Australis	bratislava	Jez-Bratislava (IP)	*L. interrogans s.s.*
Australis	bratislava	Jez-Bratislava (KIT)	*L. interrogans s.s.*
Australis	münchen	München C90 (IP)	*L. interrogans s.s.*
Sejröe	saxkoebing	Mus 24 (IP)	*L. interrogans s.s.*
Sejröe	hardjo	Hardjoprajitno (IP)	*L. interrogans s.s.*
Sejröe	roumanica	LM 294 (IP)	*L. interrogans s.s.*
Sejröe	wolffi	3705 (IP)	*L. interrogans s.s.*
Sejröe	geyaweera	Geyaweera (IP)	*L. interrogans s.s.*
Sejröe	medanensis	Hond HC (IP)	*L. interrogans s.s.*
Sejröe	recreo	380 (IP)	*L. interrogans s.s.*
Sejröe	polonica	493 Poland (IP)	*L. borgpetersenii*
Sejröe	hardjobovis	Sponselee (IP)	*L. borgpetersenii*
Sejröe	sejröe	M 84 (IP)	*L. borgpetersenii*
Sejröe	istrica	Bratislava (IP)	*L. borgpetersenii*
Sejröe	nyanza	Kibos (IP)	*L. borgpetersenii*
Sejröe	dikkeni	Mannuthi (IP)	*L. borgpetersenii*
Sejröe	gorgas	1413 U (IP)	*L. santarosai*
Sejröe	caribe	TRVL 61866 (IP)	*L. santarosai*
Sejröe	trinidad	TRVL 34056 (IP)	*L. santarosai*
Pomona	tsaratsovo	B 81/7 (IP)	*L. kirshneri*
Pomona	kunming	K 5 (IP)	*L. kirshneri*

* IP, Collection of the WHO Collaboration Centre for Leptospires, Institut Pasteur, Paris, France; KIT, Collection of the Koninklijk Institut voor de Tropen, Amsterdam, The Netherlands.

genetic methods have recently proved to be valuable for characterization of leptospira strains.

It had been shown previously that, although the sequences are very close to each other, 16S rDNA gene sequencing could allow the separation of *Leptospira* species and could be used for the comparison of different leptospira isolates [3, 10, 11]. Moreover, the partial sequencing of 16S rDNA gene amplicons (330-bp-long sequences corresponding to position 46–375 *Escherichia coli rrnB* numbering) has significant discriminatory power since almost 50% of the polymorphic positions recorded on the whole 16S rDNA gene of leptospira were localized within the first 330 bp [11].

Pulsed-field gel electrophoresis (PFGE) of large DNA fragments produced by rare-cutting restriction enzymes yield different electrophoretic patterns offering the advantage of a simple interpretation combined with a rapid result [12, 13].

In this paper, we report identification and characterization of leptospira isolates recently isolated from small rodents by phylogenetic analysis conducted from the partial 16S rDNA gene sequences and the analysis of the polymorphism generated by PFGE. Using molecular tools, this study attempts to clarify through ecological approach, the occurrence and characters of the leptospiral serovars circulating in Croatia.

METHODS

Bacterial strains studied

Most of the leptospiral reference strains used in the study are from the Collection of the WHO Collaboration Centre for Leptospires, Institut Pasteur, Paris, France. Others were from collection kept at the Koninklijk Institut voor de Tropen (KIT), Amsterdam, The Netherlands. They are listed in Table 1.

Study sites and investigation of small rodents

A total of 16 leptospiral strains (assigned as M1, M3, M10, M11, M12, M18, M27, M29, M30, M31, M33, M116, M218, M222, M261, M262) were isolated from

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Table 2. *Specification of captured small rodents, number of kidney cultures and leptospiral isolates obtained*

Species	Kidney cultures	Isolated serovars: *L. borgpetersenii* sejröe istrica	*L. interrogans* australis lora	*L. kirshneri* pomona tsaratsovo	Total	%
Apodemus agrarius	74	0	0	4	4	5·4
Apodemus flavicollis	67	0	1	1	2	2·9
Apodemus sylvaticus	42	0	0	0	0	0
Auricola terestris	1	0	0	0	0	0
Clethrionomys glareolus	23	0	0	0	0	0
Microtus agrestis	5	0	0	0	0	0
Microtus arvalis	3	0	0	0	0	0
Mus musculus	14	10	0	0	10	71·4
Total	227	10	1	5	16	7·0

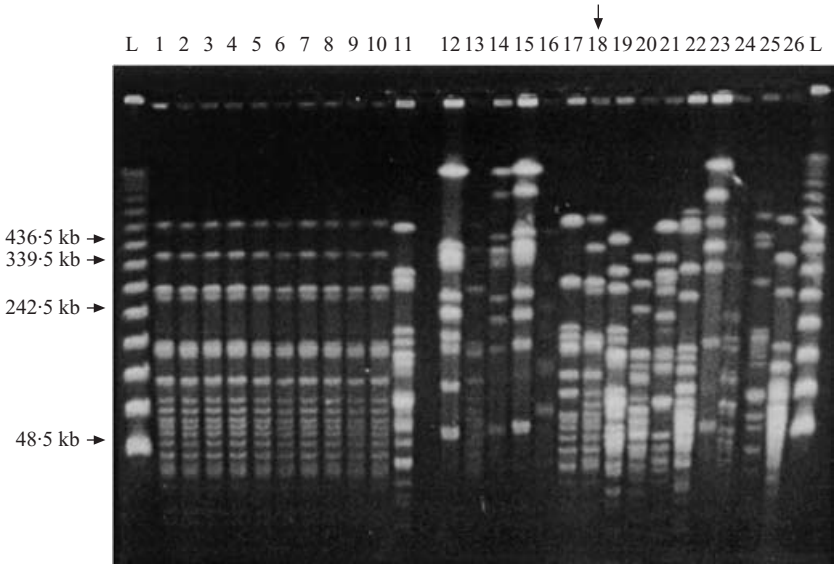

Fig. 1. PFGE of *Not*I restriction fragments of serogroup Sejröe. The digestion products were separated at 150 V for 36 h in 1 % agarose 0·5 × Tris-borate-EDTA. Pulse time was 5–30 s. Lanes 1–10, serovar istrica isolates (M1, M3, M10, M11, M12, M18, M27, M30, M31, M33); 11, serovar saxkoebing Mus 24; 12, serovar hardjo Hardjoprajitno; 13, serovar polonica 493 Poland; 14, serovar romanica LM 294; 15, serovar wolffi 3705; 16, serovar hardjobovis Sponselee; 17, serovar sejröe M 84; 18, serovar istrica Bratislava (signed with arrow); 19, serovar gorgas 1413 U; 20, serovar geyaweera Geyaweera; 21, serovar dikkeni Mannuthi; 22, serovar caribe TRVL 61866; 23, serovar medanensis Hond HC; 24, serovar recreo 380; 25, serovar nyanza Kibos; 26, serovar trinidad TRVL 34056, L, Lambda ladder PFG Marker (Biolabs).

the kidneys of 227 small rodents captured from March to October 2000 in 11 different geographical areas in inland Croatia where human and animal leptospirosis is a common disease (Karlovac, Velika Gorica, Popovaca, Kutina, Stara Gradiska, Nova Gradiska, Nova Kapela, Vinkovci, Otok, Vrbanja and Gunja). Rodents were captured using Sherman's live traps and the species were identified and separated. Animals were euthanized, aseptically dissected, and the kidney tissue was used for culture.

Culture conditions and serogroup identification

Isolates were grown in 5 ml Korthof's liquid medium [9] prior to the EMJH liquid medium [14, 15] 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 [16]. The growth was verified by counting in a Petroff–Hauser chamber. Agglutinations were performed by mixing equal quantities of a well-grown culture

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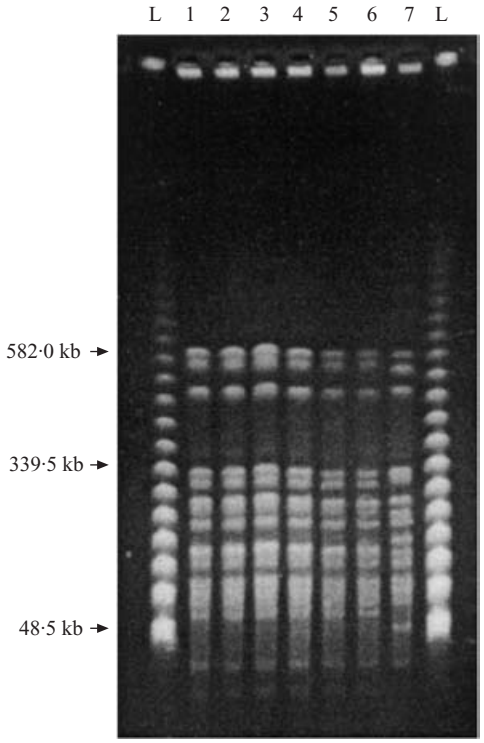

Fig. 2. PFGE of *Not*I restriction fragments of serogroup Pomona. The digestion products were separated at 150 V for 36 h in 1 % agarose 0·5 × Tris-borate-EDTA. Pulse time was 5–30 s. Lanes 1–5, serovar tsaratsovo isolates (M116, M218, M222, M261, M268); lane 6, serovar tsaratsovo B 81/7; lane 7, serovar kunming K 5. L, Lambda ladder PFG Marker (Biolabs).

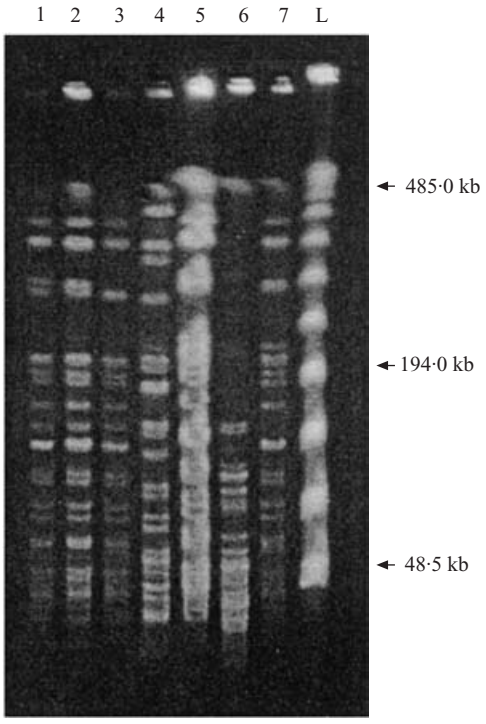

Fig. 3. PFGE of *Sgr*AI restriction fragments of serogroup Australis. The digestion products were separated at 150 V for 36 h in 1 % agarose 0·5 × Tris-borate-EDTA. Pulse time was 5–30 s. Lane 1, serovar lora isolate (M29); lane 2, serovar lora Lora; lane 3, serovar jalna Jalna; lane 4, serovar jalna Jalna (KIT); lane 5, serovar bratislava Jez-Bratislava; lane 6, serovar bratislava Jez-Bratislava (KIT); lane 7, serovar münchen München C 90. L, Lambda ladder PFG Marker (Biolabs).

(2×10^8 cells ml^{-1}) and serial dilutions of hyper-immune reference antisera produced in rabbits. The agglutination was read using dark-field microscopy.

Sequencing

The primers used to amplify the first 330 bp of the 16S rDNA gene were LEPTOA (5′-GGCGGCGCGTCTTAAACATG-3′) with positions 38–57 and LEPTOB (5′-TTCCCCCCATTGAGCAAGATT-3′) with positions 367–347 [17]. The sequencing internal primers used were LEPTOC (5′-CAAGTCAAGCGGAGTAGCA-3′) with positions 58–76 and RS4 (5′-TCTTAACTGCTGCCTCCCGT-3′) [11]. Sequencing was performed by Genome Express (Montreuil, France). Access numbers of the sequences are: M1, AY149228; M3, AY149223; M10, AY149230; M11, AY149222; M12, AY149231; M18, AY149227; M27, AY149229; M29, AY149232; M30, AY149226; M31, AY149225; M33, AY149224; M116, AY149218; M218, AY149221; M222, AY149220; M261, AY149219; M262, AY149217.

Phylogenetic analysis

The sequences obtained in this study and those available in data banks were aligned both automatically with Clustal V and manually with VSM [18]. They were analysed by distance methods, Unweighted Pair Group with Mathematical Average (UPGMA) [19] and Neighbour Joining (NJ) [20] with Mega Software [21]. The distances were calculated by the Jukes Cantor correction [20] and the tree construction was performed in a pairwise deletion procedure.

DNA extraction, enzyme digestion and serovar identification using PFGE

Preparation of the DNA was performed as previously described [13] and the plugs were stored in 0·5 M EDTA at 4 °C until used. Plugs were digested with *Not*I (Amersham) or *Sgr*AI (Bio-Rad) in accordance with the manufacturer's instructions and then slices (1–2 mm thick) were transferred to a 1 % agarose gel.

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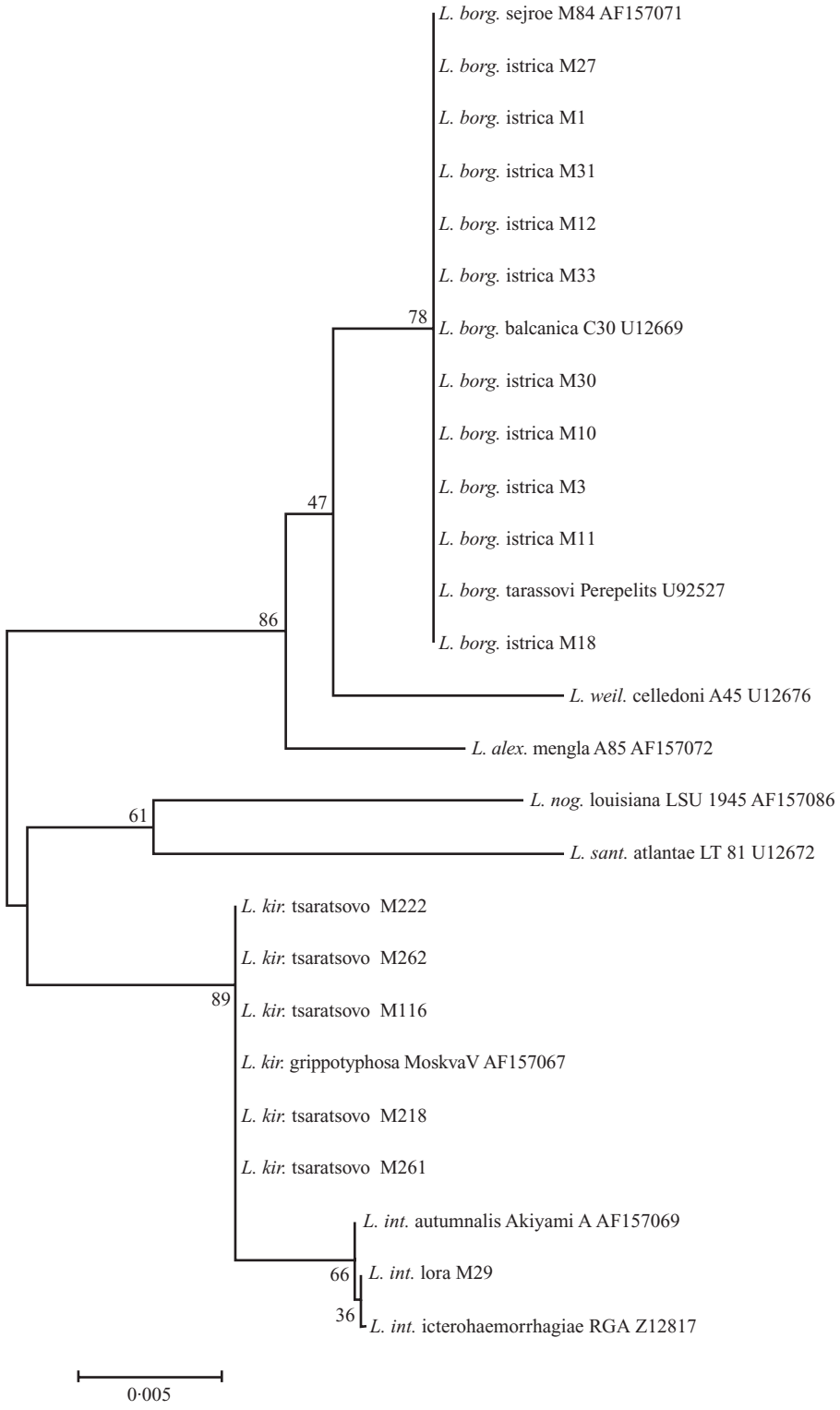

Fig. 4. Phylogenetic tree of partial 16S rDNA sequences (330 bp between positions 46 and 375 *E. coli* numbering) available from databanks and from strains determined in this study. The phylogenetic tree was drawn using the neighbour-joining method. The numbers at the branch nodes indicate the results of the bootstrap analysis.

PFGE was performed with a contour-clamped homogenous electric field system (DRII apparatus, Bio-Rad Laboratories, Richmond, CA, USA). Separation of DNA fragments was achieved with three different pulse times: 5–60 s for 35 h at 150 V; 5–30 s for 36 h at 150 V and 20–120 s for 36 h at 170 V in order to

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separate small and large DNA fragments, respectively. The sizes of DNA fragments were estimated by comparison of bands with concatemerized λ bacteriophage genomes with a size range 50–1000 kb (Lambda ladder PFG Marker (Biolabs), Beverly, MA, USA). Gels were photographed under UV transillumination after staining with ethidium bromide. DNA fragment patterns were examined visually. Isolates with identical restriction fragment patterns with two enzymes were assigned as the same leptospiral serovar.

RESULTS

To obtain a better insight of the occurrence of the leptospiral serovars in areas of inland Croatia, a total of 227 small rodents scattered into 8 species were captured in 11 different locations and investigated for the presence of leptospires. Specification of captured animals as well as leptospira isolates are listed in Table 2. From 227 kidney tissue cultures, a total of 16 leptospiral isolates were obtained from 7·0% of the captured animals: 10 from *Mus musculus*, 2 from *Apodemus flavicollis* and 4 from *Apodemus agrarius* with carrier rates of 2·9–71·4% in different species (Table 2). After a first typing according to serogroup affinities the isolates appeared to belong to serogroups Sejröe (10 isolates), Pomona (5 isolates) and Australis (1 isolate).

Intact genomic DNA from 16 leptospiral isolates was digested with restriction enzymes and submitted to PFGE. PFGE analysis of the *Not*I restriction patterns allowed us to differentiate serovars within the serogroups Sejröe and Pomona while analysis of the *Sgr*AI restriction pattern distinguished serovars into the serogroup Australis with similar *Not*I patterns. Results showed that 10 leptospiral isolates (all from *M. musculus*) appeared to have the same banding pattern which ranged in molecular size from approximately $<$50–450 kb similar to serovar istrica of the serogroup Sejröe (Fig. 1). Five isolates (4 from *A. agrarius* and 1 from *A. flavicollis*) with the same binding pattern which ranged in molecular size from approximately $<$50–650 kb were similar to serovar tsaratsovo of the serogroup Pomona (Fig. 2) and finally, 1 isolate (from *A. flavicollis*) has banding pattern with the molecular size from approximately $<$50–500 kb similar to serovar lora of the serogroup Australis (Fig. 3). Reference strains of two serovars (jalna and bratislava) from serogroup Australis belonging to different reference collections (Table 1) yielded different fingerprinting profiles (Fig. 3). Similar findings of collection heterogeneity between serovars belonging to the controversial species *L. meyeri* provided by three different collections was reported previously [11].

Partial sequencing of the 16S ribosomal gene revealed the belonging of strains to three different species, *L. borgpetersenii*, *L. kirschneri*, *L. interrogans* (Fig. 4). Otherwise, within each species the nucleotidic sequences were remarkably conserved, since all sequences exhibited 100% identity. As previously observed [11], *L. interrogans* and *L. kirschneri* clustered very close together as expected from the alignment of sequences differing by only one nucleotide. However, the phylogenetic analysis allowed to clearly assign strains to different species by both Neighbour Joining (Fig. 4) or UPGMA (data not shown) methods.

DISCUSSION

In Croatia, no small rodent survey has been carried out since 1983 [8] although leptospirosis in humans and animals continued. Borcic et al. [7] found a leptospira carrier rate in 8·9% of examined small rodents in the Sava valley (northeast Croatia) and confirmed their previous studies [8] showing that the whole area of Posavina (northeast Croatia) to be a wide natural focus of leptospires of the pomona, grippotyphosa and sejröe serovars, although it is believed that in the same area other leptospiral serovars may also find favourable conditions for their survival in nature.

Data on the leptospiral infection rates and the character of isolates from small rodents are rare in Europe. Stanko et al. [22] reported the prevalence of leptospiral antibodies in 5·0% of small rodents examined in Slovakia with the most frequently antibodies directed against the serovars grippothyphosa and sejröe. The most frequent species of rodents were *A. flavicollis*, *A. agrarius* and *Clethrionomys glareolus*. Webster et al. [23] found antibodies to leptospira in 14% of wild brown rats (*Rattus norvegicus*) in British farms and Adler et al. [24] reported the leptospira carrier rate in 12·6% of the examined small rodents in an urban environment in Switzerland. We found *A. agrarius*, *A. flavicollis* and *A. sylvaticus* are most common among captured small rodents (Table 2) which confirms previous investigations [7, 8] in Croatia. It has to be mentioned that there was no proportional rate among different species of captured animals in all study sites (data not shown). So, the lack of some species in certain regions was more probably due to random sampling (trapping) of animals to be

examined rather than actual lack of species. The findings of the present investigations showed a surprisingly high prevalence of the serovar istrica (71·4%) among the population of *M. musculus*, indicating on possible epizootic state at the capture area. This high prevalence points out the risk of heavy soil contamination by leptospires excreted in the urine of these animals. Close interaction between humans, animals, soil and water in these regions defines a considerable hazard of leptospiral infection to the local population as well as for outdoor-reared domestic animals. Moreover, the majority of *M. musculus* were captured in a hunting area (Kutina) with a large population of wild animals and a high risk of leptospirosis spreading. Sebek et al. [25] already reported that *M. musculus* could be a reservoir for the serovar sejröe. The serovar tsaratsovo were isolated from 5·4% of *A. agrarius* captured. Although it is well known that maintaining host to the serovars of Pomona serogroup is *A. agrarius*, the finding of serovar tsaratsovo is surprising since it has never been reported in Croatia. The serovar lora is also newly reported in Croatia. The serovar istrica takes place in leptospirosis transmission chain in certain parts of east Croatia as described previously [8]. It is interesting that we did not found the serovar icterochaemorrhagiae among the small rodents examined despite its undoubted role in the epidemiology of leptospirosis in humans and animals in Croatia. This confirms previous studies [8, 26], suggesting that serovar icterochaemorrhagiae has no reservoir among 'wild' mice, and its transmission occurs exclusively through rats (*R. norvegicus*). Reference serovars within the serogroup Sejroë were distributed into three species. Serovars belonging to *L. borgpetersenii* were mostly isolated from Central Europe (Bulgaria, Czech Republic, Romania, Poland). Thus, isolation of a large number of strains belonging to a serovar from *L. borgpetersenii* is not surprising. Similarly, serovars within the serogroup Pomona isolated in the same geographic region mostly belonged to the species *L. kirschneri*. Thus, identification of *Leptospira* species isolated in Croatia was in accordance with previously known geographical distribution of different serovars.

Since 1990, it has been shown that PFGE [11–13, 27–29] is usually in agreement with serotyping when considering serovar identification. This method displays many advances, objectivity, rapidity, commercially available reagents and pictures. However, the routine use of this method is still in its infancy. Difference between the patterns provides a reliable identification for each isolate by comparing each reference strain inside the serogroup. Restriction fingerprints can reveal degrees of relatedness among leptospiral strains; the more closely related the strains are genetically, the more similar their fingerprint patterns are.

In conclusion, using molecular tools we confirmed previous knowledge about occurrence of the serovar istrica in Croatia, but we reported for the first time the occurrence of the serovars tsaratsovo and lora. Results of this work are an initial step in the investigation of the molecular epidemiology of leptospirosis in Croatia. The role of different small rodents is still elusive and remains to be clarified. The relations of the reservoirs, humans and animals in the epidemiologic chain of leptospirosis in Croatia should be further studied, particularly on the molecular biology level.

ACKNOWLEDGEMENTS

We thank the Institut Pasteur which supported this work. The contribution of Dragica Zivkovic as a technical support is acknowledged.

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