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Prognostic factors for survival of canine patients infected with *Leptospira* spp.

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ABSTRACT

The objectives of this study were to describe the epizootiological and clinical features of canine patients with leptospirosis, and to determine the association between the health parameters observed and patient survival. The study population consisted of sixty patients admitted to the Department of Microbiology and Infectious diseases with Clinic, Faculty of Veterinary Medicine in Zagreb, Croatia from 2009 to 2017. The majority of the infections were caused by serogroup Pomona (50%), while serogroups Icterohaemorhagiae, Grippotyphosa, Australis and Sejroe were identified in 30%, 8.3%, 5% and 1.7% of the patients, respectively. At initial presentation, the most frequently detected organ dysfunction was renal failure (85%), followed by pulmonary (60%) and hepatic injury (58.3%). When the frequency of animals with involvement of organ systems was calculated, 31.7% had three systems involved, 46.7% had two systems involved, 13.3% had one, and 5% did not have any system involved. The mortality risk in the group that had 0, 1, 2 and 3 systems involved was 0%, 62.5%, 46.4%, and 89.5%, respectively. In the univariable analysis, several factors were univariably associated with the risk of a lethal outcome including: day of admission ($P<0.01$), sex ($P = 0.09$), *Leptospira* vaccination status ($P = 0.11$), the presence of moderate to severe acute kidney injury ($P<0.01$), pulmonary involvement ($P = 0.11$), and several other parameters. In the multivariable Cox's model, the presence of moderate and severe kidney injury was identified as a statistically significant factor associated with lower survival. The frequency of severe clinical cases of canine leptospirosis caused by the serogroup Pomona found in this study supports the need to consider inclusion of strains of this serogroup in vaccines available on the European market.

Key words: dog; leptospirosis; Pomona; survival

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Introduction

Leptospirosis is a disease of humans, various domestic animals and wildlife, caused by distinct pathogenic serovars of the *Leptospira* genus. Accurate estimation of the global frequency of both human and animal leptospirosis has been challenging due to the heterogeneity of the genus, the quality and availability of diagnostic tests, and the inconsistent clinical manifestation (HARTSKEERL et al., 2011). In addition, animal leptospirosis is not routinely monitored, which further contributes to uncertainties about the incidence and prevalence of presumptive infective serovars in different animal species.

Fifty years ago, canine leptospirosis was mainly associated with the serovars Canicola (harboured by dogs) and Icterohaemorrhagiae (maintained by rats). It is believed that long term vaccination using bivalent vaccines led to a temporary decrease in disease incidence (ANDRÉ-FONTAINE, 2006; ELLIS, 2010). The largest effect of routine vaccination has been a considerable decrease in infections caused by the serovar Canicola, probably due to reduced pathogen shedding in vaccinated animals (ANDRÉ-FONTAINE, 2006). Contrary to this, serovar Icterohaemorragie has been maintained and shed into the environment by rats. Thus, infection pressure likely remained mostly unaffected by vaccination practices in dog populations. The first reported shift in the occurrence of the infective serogroups, followed by re-emergence of canine leptospirosis, was described in the 1990's in North America. The increased incidence of severe clinical cases associated with serovars Pomona and Grippotyphosa (RENTKO et al., 1992; ADIN and COWGILL, 2000) led to the inclusion of those serovars in vaccines available there. Most of the studies conducted in Europe over the last 20 years have shown that the serogroup Icterohaemorrhagiae is still a leading cause of canine leptospirosis, but the increased frequency of clinical cases caused by serogroups Grippotyphosa, Australis, Sejroe and Pomona was also noted (GEISEN et al., 2007; ELLIS, 2010; ARENT et al., 2013; HABUS et al., 2017).

Clinical features in dogs vary from subclinical infections to severe conditions, with multiorgan involvement. The most common clinical manifestations are related to acute kidney injury (AKI) and liver impairment (SCHULLER et al., 2015). Similarly to human populations, leptospirosis pulmonary haemorrhagic syndrome (LPHS) recently emerged in dogs as a serious complication associated with high mortality rates (KLOPFLEISCH et al., 2010; KOHN et al., 2010; MAJOR et al., 2014). In addition, haemorrhagic manifestations, mostly due to disseminated intravascular coagulation, have been reported in approximately 20% of severe clinical cases (KNÖPFLER et al., 2017). Reproductive failure and ocular manifestations may also occur, although they are not as common as in some other animal species (ROSSETTI et al., 2005; TOWNSEND et al., 2006)

Canine patients admitted to veterinary clinics with suspicion of leptospirosis are regularly assessed for their condition, using a combination of observed clinical signs, haematological and biochemical blood parameters, and various imaging techniques. These parameters could then be used, *inter alia*, to make prognosis on an individual patient basis. The objectives of this study were to describe the epizootiological and clinical features of canine patients with leptospirosis at admission to a referral veterinary clinic, and to determine the association between the observed parameters and their survival.

Materials and methods

Study population. This was retrospective cohort study of canine patients with confirmed leptospirosis admitted to the Department of Microbiology and Infectious diseases with Clinic, Faculty of Veterinary Medicine in Zagreb, Croatia, in the period from 2009 to 2017. Both primary admissions, as well as the referrals from other veterinary clinics and animal shelters, were included in the study. In total, medical records were available for 60 patients.

The inclusion criteria were: a fourfold rise of titre in paired sera detected by the Microscopic Agglutination Test (MAT), single MAT titre ≥1:800, positive culture and/or PCR in blood and/or urine. Additional confirmation by PCR/isolation or fourfold rise of titre in paired sera was needed

in cases when a vaccinated animal reacted to serovars present in the vaccines available on the European market. All sera samples were tested by MAT following the standard procedure (DIKKEN and KMETY, 1978; GORIS and HARTSKEERL, 2014) using a panel of 13 *Leptospira* serovars: Grippothyphosa, Sejroe, Bratislava, Pomona, Mozdok, Canicola, Icterohaemorrhagiae, Tarassovi, Saxkoebing, Ballum, Bataviae, Poi and Hardjo type bovis. Isolates obtained by haemo- culture or urine culture were typed with rabbit antisera against the same serovars. The presumed infective serogroups in each case were determined by identifying the highest titres to one or more serovars belonging to a certain serogroup. In the case of co-agglutination resulting in equal titres against two or more serogroups, or when diagnosis was made by PCR, the aetiological serogroup was classified as undetermined. Conventional PCR was performed as described previously by MERIEN et al., 1992.

Description of clinical and laboratory parameters at admission. At the time of admission, patients had their demographic and history information recorded, and were clinically examined by the attending clinician. Laboratory and radiographic findings were obtained from available medical records and assessed using standardized criteria. Anaemia was further classified on the basis of a decrease in haematocrit (HCT) as mild (30-37%), moderate (20-29%), severe (12-19%), or very severe (<12%). Similarly, on the basis of a decrease in thrombocytes (PLT), thrombocytopenia was further classified as mild ($100\text{-}199\times10^9$/L), moderate ($50\text{-}99\times10^9$/L), severe ($25\text{-}49\times10^9$/L), or very severe ($<25\times10^9$/L).

Patients were also categorized into groups on the basis of the number of systems involved (from 0 to 3). Renal involvement was defined and graded on the basis of the IRIS system to grades II (creatinine range 141-220 μmol/L), III (creatinine range 221-439 μmol/L), IV (creatinine range 440-880 μmol/L) and V (creatinine above 880 μmol/L). Hyperbilirubinaemia with elevation of liver enzymes was used to define animals with liver involvement. Pulmonary involvement was defined as the presence of dyspnoea, abnormal lung sounds and/or indication of pulmonary involvement, as suggested by thoracic radiography.

During hospitalization all the patients were treated with intravenous antibiotics (ampicillin or amoxicillin clavulanate), fluids and H2-receptor antagonists. When needed, symptomatic treatment included antiemetics, gastroprotectants, mannitol, furosemide, and oxygen and/or analgesics. Patients with severe LPHS were also treated with prednisolone. Oral doxycycline was prescribed upon discharge in order to eliminate renal carriage. The values obtained at admission were summarized using descriptive statistics. Frequency tables were used to describe categorical variables and assess the non-survival rate in each discrete level. Medians and interquartile range were used to describe quantitative parameters in the overall population of patients and, after stratification, on the basis of their survival status. Only the parameters that were obtained on presentation, before therapy, were extracted and entered into spread sheet.

Statistical analysis A case of lethal outcome was defined as an animal that died within the first 14 days after admission to the veterinary clinic, or that was euthanized due to very a severe clinical condition and poor prognosis. Despite additional data being present for some patients, the 14-day period of interest was considered as suitable because we anticipated that the risk of lethal outcome would be highest for patients with acute illness. The number of days between initial admission to the hospital and the date of death or euthanasia was used as their survival time. Canine patients that survived and for which follow-up information was available were considered as censored at day 14 after initial admission.

In a few cases, the patient was considered as censored at the time when that patient was no longer available for further supervision by the attending clinician (n = 3) and therefore considered lost to follow-up. For statistical analysis, the patients were grouped by age (≤ 0.5 yrs, >0.5 to≤1.5 yrs, >1.5 to≤ 5 yrs, and >5 yrs) and weight categories *(*≤10 kgs, >10 to≤ 25 kgs, >25 to≤ 40 kgs and >40 kgs).

Two approaches were used to conduct univariable analysis. For categorical variables,

survival analysis based on Cox's regression was used. All factors with an overall p-value of≤0.15 on the likelihood ratio test during univariable analyses were considered as eligible for inclusion in the final regression model. For quantitative blood and biochemistry parameters, the functional relationship with lethal outcome was assessed using logistic regression based on fractional polynomials. This approach was selected since it was anticipated that a substantial proportion of blood and biochemistry parameters could have a complex relationship with the risk of mortality. Logistic regression, using fractional polynomials, was flexible enough to assess the complex relationship, and to provide a p-value for association. The overall p-value for the entire variable was used to declare if the parameter was univariably associated with the likelihood of mortality. Quantitative variables were not considered for inclusion in Cox's regression model. Instead, selected parameters were used to classify animals into groups on the basis of the involvement of certain systems (as explained above); these categorical variables were then offered for univariable analysis based on Cox's regression.

The final models were based on a manual backward selection method of evaluating prognostic factors that reached nominal significance level during univariable analysis. The statistical significance of each of the prognostic variables was evaluated using a partial likelihood ratio test, and the variable with the highest p-value was removed from the model. This process continued until all of the variables that remained in the model were statistically significant, or were considered as potentially important confounders. The choice of the final model was based on the lowest value of the Akaike information criterion (AIC). After construction of the final model, the assumption of proportional hazard was evaluated through examination of scaled Schoefiled residuals, and evaluation of the global test of hazard proportionality. The goodness of fit test was conducted using the Hosmer-Lemeshow goodness of fit test. Models were interpreted using hazard ratio. All statistical analyses were conducted using commercial software (Stata 15).

Ethical approval for this study was obtained from the Institutional Ethics Committee (251-61-44-17- 02).

Results

Leptospira testing. Sera of all the animals were tested by MAT. In 45 animals diagnosis was based solely on a fourfold rise in paired sera or single MAT titres of ≥ 1:800 against non-vaccine serogroups. An additional six dogs had a positive combination of MAT titres (≥ 1:800) at admission and positive PCR and/or

blood or urine cultures. Initial samples of nine animals were MAT negative (<800), but were found to be positive by PCR or culture. The frequency of infective serogroups in admitted patients varied over time. Overall, the most common serogroup detected in our canine patients was Pomona (50%), followed by Icterohaemorhagiae (30%), Grippotyphosa (8.3%), Australis (5%) and Sejroe (1.7%). The presumptive infective serogroup was not determined in 5% of cases. The highest titres recorded for representatives of presumptive infective serogroups were as follows: Pomona (1:204 800), Icterohaemorrhagiae (1:102 400), Grippotyphosa (1:25 800), Australis (1:6400) and Sejroe (combination of MAT titre 1:200 and positive blood PCR).

Most admissions occurred in late summer and early autumn, with September 2014 having the highest number of admitted cases (Fig. 1). When considering the number of patients admitted per month for the two most common serogroups, it appeared that Pomona cases generally peaked earlier in the season with the mode in September, whereas cases caused by Icterohaemorhagiae had a mode number of cases in October and November (Fig. 1). Interestingly, at least in this study population, there was a qualitative shift in the frequency of patients that occurred in 2014. Until that year, Icterohaemorhagiae cases were more common, but from 2014, more Pomona cases were observed in each year during the study period (Fig. 1).

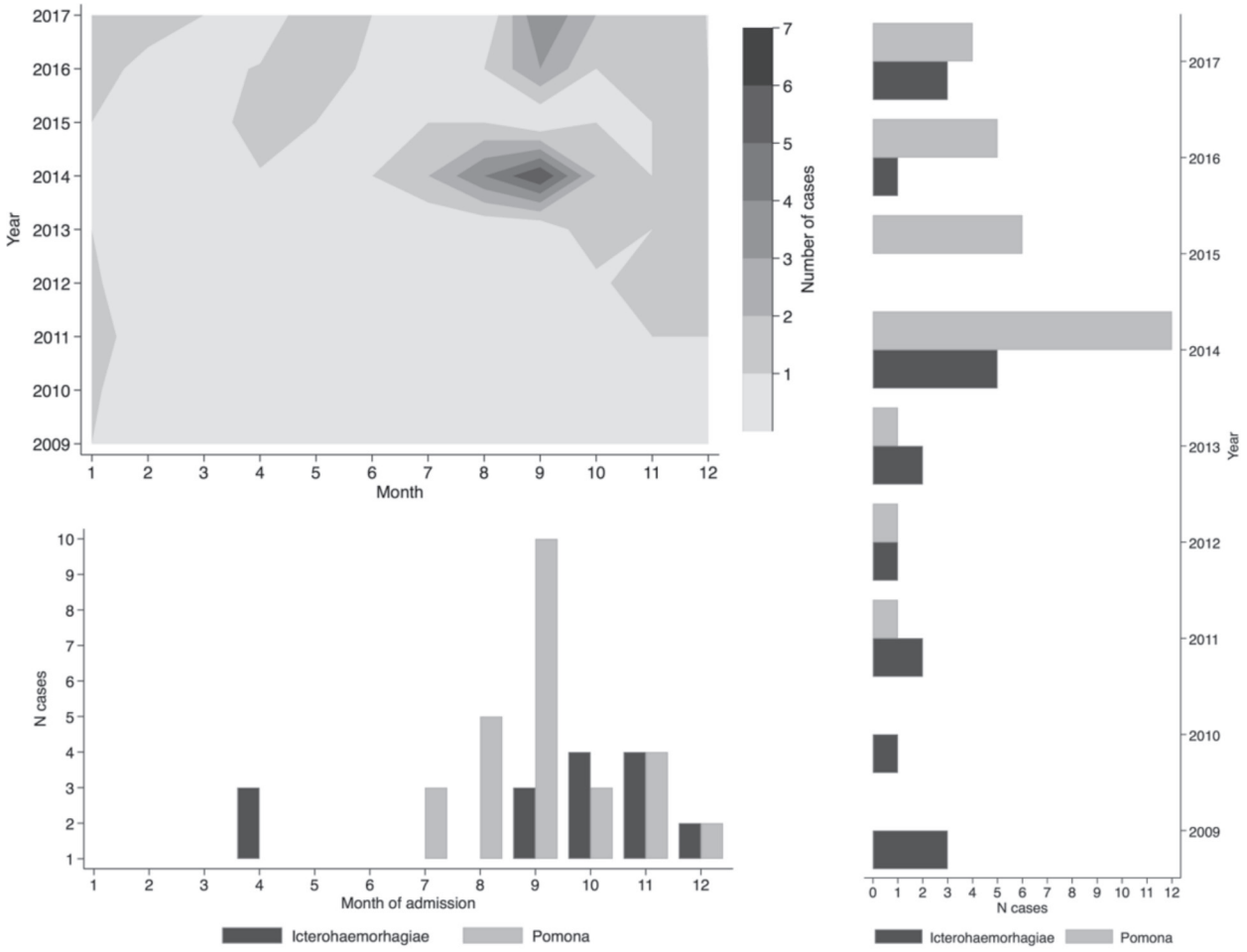

Fig. 1. Frequency distribution of 60 confirmed canine leptospirosis patients admitted to the Department of Microbiology and Infectious diseases with Clinic, Faculty of Veterinary Medicine in Zagreb, Croatia. (2009-2017), displayed as a surface plot over year-month combination. The frequency distribution of the two most common serogroups (Pomona and Icterohaemorhagiae) by year (right panel), and by month of admission (bottom left panel) is also displayed.

Description of study population. Thirty-five per cent of our canine patients were of mixed breed, and the remaining 65% were purebred, representing 21 different breeds. Male dogs, representing 60% of the study patients, were more common than females (Table 1). The mean age of the admitted patients was 5.04 years (median = 4.5 yrs; SD = 3.7 yrs), with a minimum of 2.5 months and maximum of 13 years. Descriptively, non-survivors had a lower average age (mean = 4.03, median = 3.5) than survivors (mean = 5.72, median = 6); and lower variability as measured by standard deviation (non- survivors = 3.3 yrs, survivors = 3.7 yrs). Once patients were grouped into age categories (0.5≤ yrs, >0.5 to 1.5≤ yrs, >1.5 to 5≤ yrs, and >5 yrs), the most common group of leptospirosis patients was older than 5 years of age (Table 2). Association between mortality risk and age for the two most common serogroups, as determined by logistic regression through fractional polynomials is depicted in Fig. 2. From this analysis, partial confounding between age and serogroup was evident. For this reason, it was decided that age should be used as a preferred variable when multivariable survival models were constructed, rather than serogroup.

The mean weight of admitted patients was 20.4 kgs, and varied between a minimum of 2 kgs and maximum of 54 kgs (median = 19.2, SD = 12.7 kgs). Once the patients had been classified into weight categories, we could see very similar

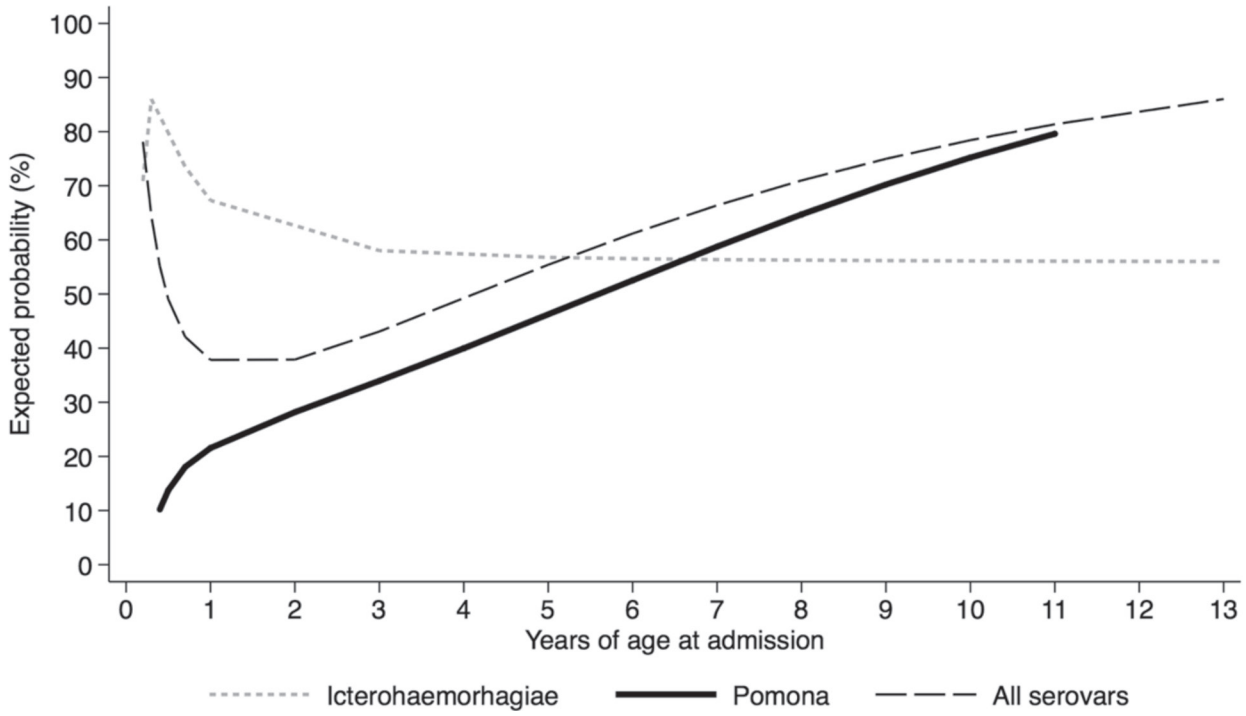

Fig. 2. Expected probability of non-survival in 60 canine patients with leptospirosis based on the two most common serogroups, and data for all patients. Probability was estimated in three separate logistic regression models where age was modelled using fractional polynomials.

representation of medium (35%), mini (31.7%) and large dogs (26.7%), whereas only 6.7% of the patients weighed more than 40 kg. Table 1 provides further details about the classification of patients into four distinct groups based on their weight at admission, vaccination status and day of admission. When patients with the two most frequent presumptive serogroups were compared relative to their vaccination status, it was interesting to note that the frequency of infection with Icterohaemorrhagiae and Pomona serogroup was similar in the unvaccinated group, with 15 and 19 patients, respectively. In contrast, the frequency of infection with Pomona serogroup (n = 8) in the vaccinated patients was relatively higher than infection with Icterohaemorrhagiae (n = 2). Moreover, both of these dogs were puppies (<0.5 y) that had been vaccinated within a year previously, but had received only a single, initial dose of vaccine. The majority of animals (95%) were hospitalized. The mean duration of hospitalization was 4 days (median = 3, SD = 3.1 days), and varied between 1 and 20 days.

Clinical signs and organ manifestations. Lethargy was the most common presenting clinical sign (97%), followed by different levels of anorexia (93.4%), dehydration (70%), vomiting (65%) and abdominal discomfort (60%). Dyspnoea and abnormal lung sounds were found in 60% and icterus in 53% of the patients. Upon admission, 45% of the patients had a body temperature within the expected range (38-39.4 °C), 26.7% were febrile (≥39.5 °C) and 26.7% hypothermic (≤37.9 °C). Interestingly, high fever and clinical icterus was observed in only 13% (n = 8) of the admitted patients. Pregnancy loss with vulvar discharge was the primary cause of admission for one patient. Preoperative assessment of this patient revealed additional renal and hepatic lesions. All clinical signs observed during examination and the non-survival rate for each level of the variable are listed in Table 3. Table 4 lists the haematological

Table 1. Description of epidemiological parameters in 60 canine patients with leptospirosis recorded at the time of admission

Parameter	Level	Overall		Non-survivors	
		n	%	n	%
Presumptive serogroup	Australis	3	5.0	3	100.0
	Grippotyphosa	5	8.3	2	40.0
	Icterohaemorrhagiae	18	30.0	12	66.7
	ND*	3	5.0	3	100.0
	Pomona	30	50.0	15	50.0
	Sejroe	1	1.7	1	100.0
Sex	Female	24	40.0	12	50.0
	Male	36	60.0	24	66.7
Weight category	Mini (< = 10 kgs)	19	31.7	15	78.9
	Medium (>10 < = 25 kgs)	21	35.0	13	61.9
	Large (>25 - <-40 kgs)	16	26.7	5	31.2
	Giant (>40 kgs)	4	6.7	3	75.0
Days sick at admission	< = 2 days	15	25.0	6	40.0
	>2-< = 5 days	31	51.7	25	80.7
	>5 days	12	20.0	4	33.3
	Missing	2	3.3	1	50.0
Vaccinated (past year)	No	41	68.3	27	69.2
	Yes	13	21.7	5	33.3
	Missing	6	10.0	4	66.7

*ND - not determined

and serum biochemistry parameters from samples obtained at the time of admission in all patients, and stratified by their survival status. The most frequent CBC abnormalities on initial evaluation were thrombocytopenia (63.4%), leucocytosis (55%) and anaemia (46.7%). In the majority of patients, both anaemia and thrombocytopenia were classified as mild to moderate (Table 5). The most frequent biochemical findings were elevation in serum urea (90%) and creatinine levels (85%). Hyperbilirubinaemia was present in 72% of patients. Among patients with available measurements, increases in alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase activities were detected in 76.9%, 53.6% and 57.8% of the dogs, respectively. C-reactive protein (CRP) concentrations measured in approximately half of the patients (29/60) were elevated in 96.5% of dogs. In the group of patients with available measurements, elevated levels of phosphorus, sodium and potassium were detected in 67.5% (n total = 40), 3.4% (n total = 29), and 6.1% (n total = 33) of patients, respectively. Hyponatraemia and hypokalaemia were observed in 27.6% and 36.4% of patients, respectively. When parameters were compared between animals with different survival status, some simple trends could be noted (Table 4). For instance, non-survivors had higher median values for urea, creatinine, creatinine phosphokinase, amylase, lipase, phosphorus, potassium, bilirubin, glucose, CRP and ALP, albeit with high variability for most parameters. Non-survivors had slightly lower mean levels of HCT and sodium. Nonetheless, a simple comparison of averages was deemed insufficient to capture complex non-linear relationships.

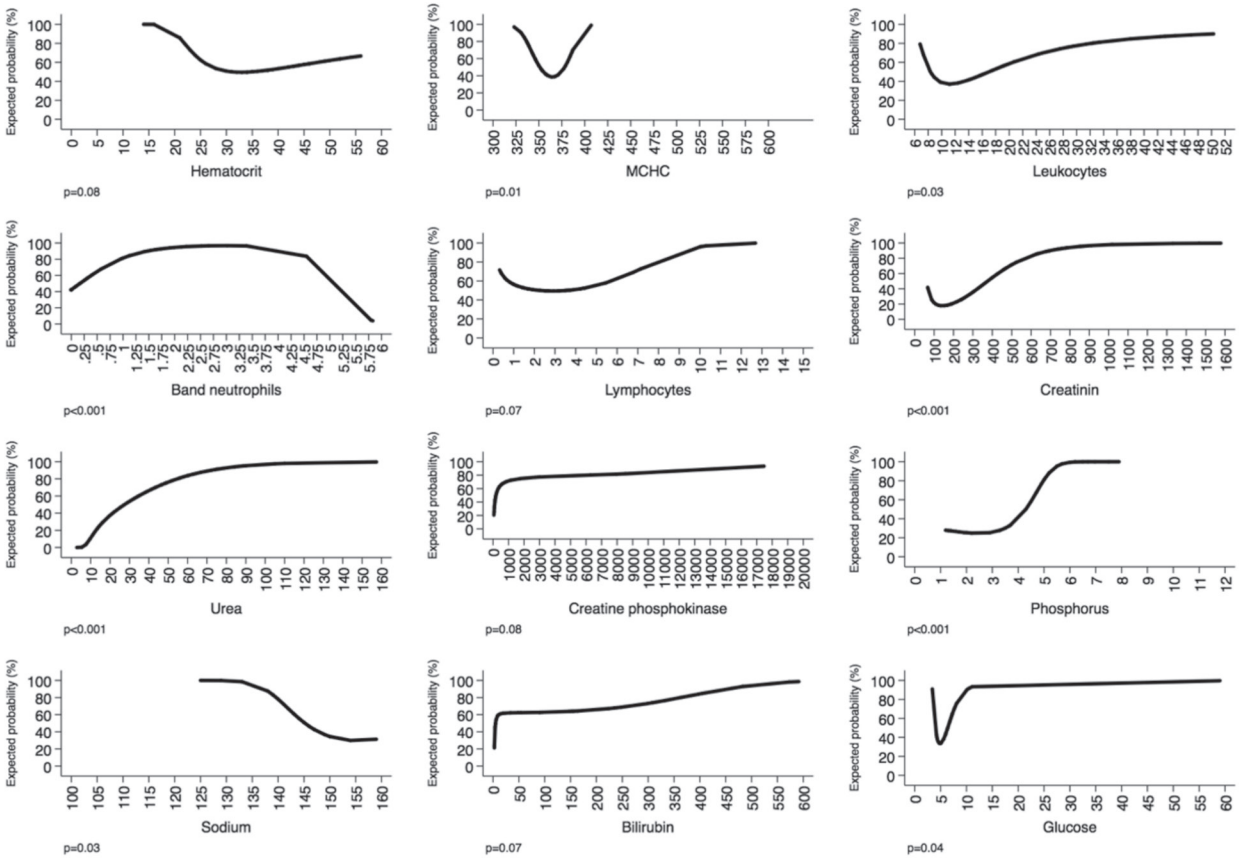

Fig. 3. Expected univariable association between survival status and significant haematology and serum biochemistry parameters obtained on the basis of fractional polynomials logistic regression, in 60 canine patients with leptospirosis and expressed on a probability scale

When the frequency of animals with involvement of organ systems was calculated, 31.7% (n = 19) had three systems involved, 46.7% (n = 28) had two systems involved, 13.3% (n = 8) had one, and 5% (n = 3) did not have any system involved (Table 5). The mortality risk in the group that had 0, 1, 2 and 3 systems involved was 0%, 62.5%, 46.4%, and 89.5%, respectively. Overall, the most frequently detected organ dysfunction was renal failure (n = 51, 85%), followed by pulmonary (n = 36, 60%) and hepatic injury (n = 35, 58.3%). In the group of animals that had multi-organ involvement we found various combinations of affected organs; kidney/liver/lungs (n = 19, 31.7% of the study population), kidney/lungs (n = 13, 21.7%), kidney/liver (n = 13, 21.7%) and liver/lungs (n = 2, 3.3%). When the AKI scores of Grade III, IV or V were considered as an indication of moderate to severe AKI (msAKI), 73.3% of patients were classified in this group. In the latter group, 72.7% did not survive, as opposed to the group without such injury, where 20% of patients did not survive (Table 5). Overall mortality risk in this study population was 60%.

Statistical analysis. The univariable associations that were significant at P<0.15 are provided in Table 6. During the model building process, it was observed that serogroup and weight were confounders for association between age and survival. Because of limited number of observations, the age category was selected as the variable for entry into the final model. Similarly, age was partially confounded by sex. Upon descriptive examination it was observed that there was approximately equal distribution of sex in all age categories, except in the group

of dogs older than 5 years of age, in which ~71% of dogs were male. In this study, age was also a partial confounder for association between day of disease at admission and survival. When examined descriptively, the majority of patients younger than 6 months (75%), and older than 5 years of age (60.7%) were admitted between days 2 and 5 of the disease.

Table 2. Cross-tabulation of presumptive infective serogroup, age category and survival in 60 canine patients with leptospirosis

		Age group (years)				
Serogroup		<0.5	0.5-1.5	1.5-5	>5	Total
	Australis	0	1	0	2	3
	Grippotyphosa	0	1	2	2	5
	Icterohaemorrhagiae	6	4	3	5	18
	ND*	0	0	1	2	3
	Pomona	2	3	9	16	30
	Sejroe	0	0	0	1	1
Total (n)		8	9	15	28	60
Total (%)		13.3	15	25	46.7	
Non-survivors (%)		62.5	44.4	46.7	71.4	60

*ND - not determined

Table 3. Description of clinical parameters in 60 canine patients with leptospirosis recorded at the time of admission

		Overall		Non-survivors	
Explanatory variable	Level	n	%†	n	%‡
Lethargy	no	2	3.3	0	0
	yes	58	96.7	36	62.1
Anorexia	no	4	6.7	1	25.0
	yes	46	76.7	31	67.4
	missing	10	16.7	4	40.0
Weakness	no	25	41.7	12	48.0
	yes	30	50.0	19	63.3
	unable to stand	5	8.3	5	100.0
Emesis	no	21	35.0	10	47.6
	yes	39	65.0	26	66.7
Feces	normal	33	55.0	17	51.5
	diarrhoea	10	16.7	8	80.0
	mucous diarrhoea	8	13.3	5	62.5
	bloody diarrhoea	2	3.3	2	100.0
	obstipation	5	8.3	4	80.4
	missing	2	3.3	0	0.0

Table 3. Description of clinical parameters in 60 canine patients with leptospirosis recorded at the time of admission (continued)

Explanatory variable	Level	Overall		Non-survivors	
		n	%†	n	%‡
Icterus	no	28	46.7	15	53.6
	yes	32	53.3	21	65.6
Dehydration	no	17	28.3	5	29.4
	yes	42	70.0	30	71.4
	missing	1	1.7	1	100.0
Urine quantity	normal	35	58.3	17	48.6
	anuria	3	5.0	3	100.0
	oliguria	10	16.7	8	80.0
	polyuria	6	10.0	3	50.0
	incontinence	2	3.3	1	50.0
	missing	4	6.7	4	100.0
Urine colour	normal	31	51.7	17	54.8
	intense yellow	9	15.0	3	33.3
	dark	5	8.3	3	60.0
	missing	15	25.0	13	86.7
Abdominal discomfort	no	23	38.3	10	43.5
	yes	36	60.0	26	72.2
	missing	1	1.7	0	0
Respiratory clinical signs	no	23	38.3	11	47.8
	yes	36	60.0	25	69.4
	missing	1	1.7	0	0
Thorax radiography	negative	14	23.3	5	35.7
	positive	31	51.7	21	67.7
	missing	15	25	10	66.7
Ultrasound	negative	5	8.3	4	80.0
	positive	21	35.0	15	71.4
	missing	34	56.7	17	50.0
Imidocarb dipropionate*	no	42	70	27	64.3
	yes	15	25	8	53.3
	missing	3	5	1	33.3
Concurrent diseases§	no	38	63.3	22	57.9
	yes	21	35.0	14	66.7
	missing	1	1.7	0	0

* Prior treatment with Imidocarb dipropionate occurred in some patients due to initial suspicion of babesiosis. § Most frequent concurrent disease was pancreatitis. † Percentage of total study population. ‡ Percentage mortality and euthanasia at this level of the variable

Table 4. Description of quantitative haematology and serum biochemistry parameters in 60 canine patients with leptospirosis recorded at the time of admission

	Total		Survivors			Non-Survivors		
Parameter (Referent range)	Median	IQR	Median	IQR	n	Median	IQR	n
Erythrocytes (5.5-8.5×10^{12}/L)	6.0	2	5.9	2	23	6	2.2	35
Haematocrit (37-55%)	38.0	14	36.5	11.5	24	38	18	35
Leucocyte (6-17×10^{9}/L)	18.5	14.7	15.6	5.6	24	20.7	15.3	35
Band neutrophils	0.2	1.6	0	0.3	23	0.4	1.9	33
Segmented neutrophils	13.1	8.7	12.3	4.2	24	16.2	10.1	33
Lymphocytes	2.6	2.5	2.5	2.2	24	2.6	3.2	33
Platelets (200-700×10^{9}/L)	155.0	174	164.5	204	24	130	140	35
Total protein (55-75 g/L)	62.0	15	61.5	12.5	24	63.5	18	34
Albumin (26-33 g/L)	26.0	7	27	5	24	25	8	34
Urea (3.3-8.3 mmol/L)	41.2	38.6	21.6	24.6	24	51.2	33.3	35
Creatinine (44-140 mmol/L)	455.0	521	229.5	201	24	678	361	35
ALT (-88 U/L)	99.5	166	69.5	152	22	105.5	181.5	32
AST (-82 U/L)	103.0	184	82.5	264	18	110	177	27
GGT (-6 U/L)	7.0	11	6.5	11	12	7	10	21
AP (20-156 U/L)	440.0	630	205	409	13	478	820	26
CPK (-160 U/L)	407.5	980	200	252	14	611	2535	20
Bilirubin (-8.6 µmol/L)	71.5	234	32.7	87.3	17	147	299	29
Amylase (-1600 U/L)	2010.0	2240	1028.5	19	2	2468	2479	10
Lipase (13-200 U/L)	409.0	926	193.5	480	10	642	1377	23
Phosphate (0.8 -1 mmol/L)	4.0	3.65	2	1.9	17	5.2	2.8	23
Sodium (140-155 mmol/L)	144.0	8	146	7	11	140.5	7	18
Potassium (3.6 - 5.8 mmol/L)	4.0	1	3.6	0.6	13	4.15	1.3	20
Glucose (3.6 -6.5 mmol/L)	6.0	2.2	5.9	1.9	17	6.5	2.25	20
CRP (0-10.7 mg/L)	121.0	106.5	100.6	100.8	12	124.6	105.7	17
PT	7.2	9.1	11.3	11.27	8	7	5.3	8
aPTT	13.35	67.55	12.5	84.35	11	16.05	31.75	12

In the univariable logistic regression conducted using fractional polynomials, several blood and blood biochemistry factors were associated with the risk of mortality in leptospirosis patients. As anticipated, such associations had different degrees of non-linearity on a probability scale, and were therefore presented as expected values, predicted by the logistic regression model (Fig. 3), with overall p-values based on the entire model. It is worth pointing out that the risk of mortality was univariably associated with low values of haematocrit and sodium, low and high values of leukocytes and glucose levels, and high values of urea, creatinine, creatine phosphokinase, bilirubin and phosphorus (Fig. 3). It was *a priori* decided that such parameters would not be considered for inclusion in the final model. Instead, the selected parameters, in combination with clinical findings and imaging, were used to classify patients as patients with kidney injury, pulmonary and hepatic involvement. Such variables were then considered for inclusion in the final model.

Table 5. Classification of 60 canine patients with leptospirosis into distinct groups based on the level of anaemia, thrombocytopenia, acute kidney injury, and hepatic and pulmonary involvement

		Overall		Non-survivors	
Parameter	Level	n	%	n	%
Anaemia	No anaemia	31	51.7	19	61.3
	Mild	16	26.7	7	43.7
	Moderate	9	15.0	6	66.7
	Severe	2	3.3	2	100.0
	Very Severe	1	1.7	1	100.0
	Missing	1	1.7	1	100.0
Thrombocytopenia	None	21	35.0	11	52.4
	Mild	22	36.7	13	59.1
	Moderate	13	21.7	9	69.2
	Severe	2	3.3	2	100.0
	Very Severe	1	1.7	0	0.0
	Missing	1	1.7	1	100.0
AKI*	No injury	8	13.3	2	25.0
	Grade II	7	11.7	1	14.3
	Grade III	14	23.3	6	42.9
	Grade IV	24	40.0	20	83.3
	Grade V	6	10.0	6	100.0
	Missing	1	1.7	1	100.0
Hepatic	No	23	38.3	12	52.2
	Yes	35	58.3	23	65.7
	Missing	2	3.3	1	50.0
Pulmonary	No	24	40.0	10	41.7
	Yes	36	60.0	26	72.2

*AKI - acute kidney injury

The final Cox's regression model, based on epidemiological and clinical parameters at admission, is listed in Table 7. Generally, moderate to severe kidney injury and level of weakness were the two parameters that remained significant in the model after adjusting for the age effect. In addition, we decided to keep the variable which indicated pulmonary involvement in the model, although it did not reach statistical significance. This was in order to evaluate whether this variable would have a confounding effect on any of the other clinically relevant variables in the model. It did not. The final model did not contain any time-varying coefficients, since the global test of proportionality of hazards indicated that the proportionality assumption was satisfied (Table 7). Scaled Schoefield residuals suggested that AKI might have some time varying effect, with hazard of mortality increasing over time in these animals. However, this investigation was not pursued further because of the low number of observations.

Table 6. Univariable association between mortality rate, and clinical and epidemiological parameters in 60 canine patients with leptospirosis recorded at the time of admission and estimated by the proportional hazard model

Variable	Level	HR	95% confidence interval		P
Serovar	Pomona	-	-	-	0.11*
	Icterohaemorrhagiae	1.87	0.84	4.18	0.13
	ND**	3.14	1.13	8.75	0.03
	Others	2.13	0.83	5.52	0.12
Day at admission	<2 days	-	-	-	0.008*
	2-5 days	2.34	0.95	5.76	0.064
	>5 days	0.61	0.17	2.15	0.44
Age category	<0.5 yrs	-	-	-	0.16*
	0.5-1.5 yrs	0.46	0.12	1.71	0.25
	1.5-5 yrs	0.51	0.16	1.62	0.25
	>5 yrs	1.12	0.42	2.99	0.82
Weight category	< = 10 kgs	-	-	-	0.08*
	10-25 kgs	0.63	0.30	1.34	0.23
	25-40 kgs	0.29	0.10	0.80	0.02
	>40 kgs	0.63	0.18	2.19	0.47
Temp (C)	Linear	0.77	0.58	1.02	0.07
Weakness	No	-	-	-	0.01*
	Yes	1.37	0.66	2.82	0.40
	Cannot stand	7.15	2.32	21.95	0.001
Sex	Female	-			0.09*
	Male	1.79	0.90	3.61	0.09
Lepto vaccinated	No	-	-	-	0.11*
	Yes	0.42	0.16	1.07	0.070
	Missing	1.18	0.41	3.37	0.759
Dehydration	no	-	-	-	0.007*
	yes	3.15	1.22	8.16	0.02
Radiograph -thorax	negative	-	-	-	0.03*
	positive	2.6	0.97	6.95	
Radiograph-abdomen	negative	-	-	-	0.052*
	positive	2.03	0.98	4.21	0.057
Kidney injury	AKI*** <3	-	-	-	<0.01*
	AKI*** = >3	4.32	1.32	14.18	
Pulmonary	no	-	-	-	0.11*
	yes	1.78	0.86	3.72	0.12
Hepatic	no	-	-	-	0.32*
	yes	1.41	0.70	2.83	0.33

* P-value determined by partial likelihood ratio test intended to assess the significance of the entire variable; **ND - not determined; *** AKI - acute kidney injury

Table 7. Association between mortality and clinical/epidemiological parameters recorded at the time of admission in 60 canine patients with leptospirosis estimated by the multivariable proportional hazard model

Level	HR	95% confidence interval		P	P*
<0.5	-	-	-	-	0.34
>0.5-1.5	1.8	0.4	8.1	0.4	
1.5-5	1.1	0.3	4.1	0.9	
>5	2.1	0.7	6.8	0.2	
no	-	-	-	-	0.02
yes	4.0	1.2	13.6	0.03	
no	-	-	-	-	0.18
yes	1.7	0.7	3.9	0.2	
no	-	-	-	-	0.04
yes	0.8	0.3	1.9	0.61	
severe	4.0	1.1	15.7	0.02	

N observations = 59; Overall LR P<0.01; GOF test (LR-based with 4 df) P = 0.85; Global test of proportional hazard assumption P = 0.25; * Partial likelihood ratio test conducted to assess the significance of the entire variable

Discussion

General trends. This study shows an overview of 60 canine patients, diagnosed and treated for leptospirosis over a period of nine years. Associations between parameters observed at admission and animal survival were investigated. One of the most important findings of this study was the high frequency of severe clinical cases caused by the serogroup Pomona. The re-emergence of canine leptospirosis combined with a potential serovar shift was reported throughout Europe (GEISEN et al., 2007; ELLIS, 2010). Increased exposure to serogroups Grippotyphosa, Australis and Sejroe detected in those studies resulted in inclusion of representative serovars to new vaccines available on the European market. Although some authors also reported increased exposure to serogroup Pomona (MODRIĆ et al., 1987; KOHN et al., 2010; CLAUS et al., 2008; ŠTRITOF-MAJETIĆ et al., 2012) the conclusion was drawn that there was not enough evidence to support inclusion of Pomona in European vaccines. Since that time, higher numbers of Pomona cases in Greece (ARENT et al., 2013), Germany (KNÖPFLER et al., 2017) and Croatia (HABUS et al., 2017) have been reported. In this study a quantitative and qualitative shift in the frequency of admitted patients occurred in 2014. The highest peak in overall incidence recorded that year was probably associated with extreme weather conditions, as described in previous publications (HABUS et al., 2017). However, the frequency of infective serogroups also changed over the study period, with Pomona being more frequent in the later part of the study period, and Icterohaemorrhagiae during the initial period. Descriptively, it appeared that Pomona cases generally peak earlier in the season, with most clinical cases in September, whereas cases caused by Icteroheamorhagiae were more common in October and November. In addition, although half of the patients in this study were infected with serogroup Pomona, Icterohaemorrhagie was considered as the main aetiological agent in puppies (75% of the cases). All the observed findings could be a reflection of different sources of infection and transmission pathways. The main reservoir of serovar Icterohaemorrhagiae is the rat, the most common peridomestic rodent, and exposure to this serovar could likely occur in the proximity of human settlements at any time of the

year. Restricted outdoor activity, that is expected in young unvaccinated puppies, will therefore not affect the infection pressure with the latter serovar. In contrast, serovars from the serogroup Pomona are maintained by swine and various species of wild small rodents, predominantly the striped field mouse (*i.e. Apodemus agrarius*), one of the main reservoirs of Pomona already documented in Croatia (BORČIĆ et al., 1986; TURK et al., 2003). It is therefore possible that adult dogs with increased outdoor activity could be more exposed to wild mice or a contaminated environment, particularly in periods when climate conditions favour *Leptospira* spp. survival. In this study, the descriptive results suggested that the majority of cases caused by the serogroup Pomona were indeed patients older than 1.5 years, whereas cases caused by the serovar Icterohaemorhagiae were almost equally distributed across different age categories (Table 5). However, this needs to be explored in future, targeted studies.

Cases at admission. Clinical manifestations of canine leptospirosis are protean, and range from subclinical to severe conditions accompanied with high mortality rates. In mild and moderate cases of leptospirosis clinical signs withdraw quickly, sometimes even without treatment (ELLIS, 2015). Those cases are likely to remain undiagnosed. Veterinarians are more inclined to consider leptospirosis in severely affected animals, especially if they present with clinical signs they consider typical of the condition. Thus, the results of this study could be biased towards more severe clinical cases.

Fever and icterus, with or without bleeding tendencies, were once considered typical clinical signs of canine leptospirosis (FAINE et al., 1999). In this study, icterus alone was present in approximately half of the patients, but in combination with high fever in only eight animals.

Recent studies have reported a change in clinical manifestation, with the emphasis on an increasing incidence of severe cases due to the LPHS (GOLDSTEIN et al., 2006; KOHN et al., 2010; KLOPFLEISCH et al., 2010; MAJOR et al., 2014). Accordingly, confirmatory testing for leptospirosis is now suggested in all cases of renal, hepatic or pulmonary injury, especially when accompanied by typical CBC abnormalities (SCHULLER et al., 2015). It has been hypothesized that the change in clinical manifestation could be associated with the shift in aetiological serogroup. This theory, however, could not be confirmed in this or other similar studies (GOLDSTEIN et al., 2006; GIESEN et al., 2007)."

Statistical analysis and prognostic factors for survival. The mortality rates observed in this study were high, particularly in patients that had evidence of three systems involved, which was almost a third of our study population. These high rates could likely be attributed to the fact that the clinic included in the study is the referral clinic for infectious diseases, mostly dealing with more severe cases. In addition, the high mortality rate could be attributed to the lack of haemodialysis, which could facilitate survival of severely azotemic patients until the recovery phase begins (ADIN et al., 2000).

Despite the low number of patients, some interesting trends in factors important for survival were detected during the different stages of the statistical analysis. The high percentage of male dogs at admission is in concordance with some previous studies (MAJOR et al., 2014; AZÓCAR-AEDO and MONTI, 2016), and could be due to the higher exposure associated with specific male behaviour. However, in univariable analysis, the hazard of lethal outcome was also higher than in female dogs, although this association did not reach statistical significance at the level of $P<0.05$ (Table 6). Interestingly, animals admitted between the 2nd and 5th days of the disease had lower survival rates than animals admitted in the first 2 days, or animals admitted after the fifth day. This could likely be due to a number of factors, including severity of clinical signs, and early detection and the reaction of owners. In general, admission early in the course of the disease was beneficial for the survival of patients. Vaccination of patients against *Leptospira* in the year before admission had the tendency to have a positive effect on survival. However, the effect of vaccination could not be fully explored in

the final model, since this model likely contained intervening variables for association between vaccination and survival.

In the final model, the presence of moderate or severe AKI was associated with the risk of dying that was four times higher than in animals without such injuries, after adjusting for other factors including age, different level of weakness, and pulmonary involvement. The presence of pulmonary involvement increased the risk of dying 1.7 times, but the association could not be declared as statistically significant at the level of $P<0.05$. Leptospirosis pulmonary haemorrhagic syndrome is the most lethal manifestation in humans (TREVEJO et al., 1998) and has already been described as a poor prognostic factor in dogs (KOHN et al., 2010). In this study, the lack of statistically significant association could be attributed to a number of factors which likely include the non- specific definition of pulmonary involvement. Pulmonary changes were not ranked, and milder forms of pulmonary involvement were also included in our definition. To illustrate this point further, positive radiographs of the thorax were a statistically significant risk factor for mortality. However, we opted not to consider this variable for the final model because it had a high proportion of missing observations. In addition, dyspnoea and/or abnormal radiographic findings could be caused by acidosis, pain and fluid overload. Such a definition likely decreased the magnitude of the estimated hazard ratio and statistical power. Any gross pathology or histopathological findings were also not considered in this study because of our inclusion criteria that were reflective of the research question. In future studies, more stringent and specific definitions should be used.

Conclusion

There seemed to be a shift in the predominant serogroups of admitted patients that occurred in 2014. The frequency of severe clinical cases caused by serogroup Pomona in the later part of the study period suggests that inclusion of strains from this serogroup in vaccines available on the European market should be given serious consideration. In addition, several variables were associated with the risk of lethal outcome in the univariable analysis. After adjustment for the age effect and other factors in the statistical model, the presence of moderate and severe kidney injury was identified as a statistically significant factor associated with lower survival. Further work is necessary to explorethe prognostic value of the quantitative variables that showed a complex relationship with survival.

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SAŽETAK

Ciljevi ovoga istraživanja bili su opisati epizootiološke i kliničke značajke pasa oboljelih od leptospiroze te utvrditi povezanost promatranih parametara i stupnja preživljavanja. Istraživanje je provedeno na 60 pasa zaprimljenih na Zavod za mikrobiologiju i zarazne bolesti s klinikom Veterinarskoga fakulteta u Zagrebu, u razdoblju od 2009. do 2017. godine. Većina utvrđenih infekcija u pasa bila je uzrokovana serološkom skupinom Pomona (50 %), dok su serološke skupine Icterohaemorhagiae, Grippotyphosa, Australis i Sejroe uzrokovale bolest u 30 %, 8,3 %, 5 % i 1,7 % pacijenata. Najčešća su klinička očitovanja bila akutna bubrežna (85 %) i jetrena lezija (58,3 %) te disfunkcija respiratornog sustava (60 %). Od ukupnog broja zaprimljenih pacijenata, u njih 31,7 % utvrđeno je oštećenje svih triju organskih sustava, u 46,7 % oštećenje dvaju organskih sustava, u 13,3 % pacijenata oštećenje jednoga organskog sustava, dok u 5 % pasa nije utvrđeno oštećenje organa. Smrtnost u skupinama u kojima je utvrđeno oštećenje 0, 1, 2 i 3 organska sustava bila je 0 %, 62,5 %, 46,4 % i 89,5 %. Univarijabilnom analizom utvrđeno je nekoliko čimbenika povezanih s povećanim rizikom od smrti: trajanje bolesti pri prijemu na kliniku ($P < 0,01$), spol ($P = 0,09$), cijepni status ($P = 0,11$), umjereno do teško akutno oštećenje bubrega ($P < 0,01$), respiratorna disfunkcija ($P = 0,11$). Multivarijabilnim Coxovim modelom prisutnost umjerenog do teškog oštećenja bubrega identificirana je kao statistički znakovit čimbenik povezan s nižim preživljavanjem. Povećana incidencija teških kliničkih oblika uzrokovanih serološkom skupinom Pomona, koja je utvrđena u ovom istraživanju, naglašava potrebu uključivanja ovih sojeva u cjepiva koja su dostupna na europskom tržištu.

Ključne riječi: pas; leptospiroza; Pomona; preživljavanje