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A Retrospective Serological Study on *Leptospira* spp. Exposure and *Brucella canis* in Dogs from Croatian Animal Shelters

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Abstract

This retrospective cross-sectional study investigated exposure to *Leptospira* spp. and *Brucella canis* in dogs housed in two animal shelters in Croatia. Archived serum samples from 375 dogs from the Dumovec shelter (Zagreb County) and the Friends Čakovec shelter (Međimurje County) were tested. Specific antibodies against *Leptospira* were detected by the microscopic agglutination test (MAT) using a panel of ten pathogenic serovars, while *B. canis* antibodies were screened using a rapid immunochromatographic assay. Associations with shelter, sex, age, serovar distribution, and antibody titers were statistically evaluated. Overall, 128/375 dogs (34.1%) were seropositive to at least one *Leptospira* serovar. Seropositivity was 29.6% in Dumovec and 36.1% in Čakovec, with no significant difference between shelters. No significant association was found between overall seropositivity and sex or age. Before adjustment for recent vaccination, the most frequent MAT reactions were directed against antigens representing Icterohaemorrhagiae, Australis, Canicola, Pomona, and Grippotyphosa. In a vaccination-related sensitivity analysis, MAT reactivity to antigens representing Australis, Pomona, and Grippotyphosa remained frequent, although this pattern should be interpreted cautiously because complete vaccine-product information was not consistently available. Most antibody titres were low to moderate, more consistent with previous exposure rather than with acute infection. No antibodies against *B. canis* were detected in any samples. These findings indicate substantial environmental exposure of shelter dogs in Croatia to pathogenic leptospires and support their role as sentinels of zoonotic risk. The predominance of MAT reactivity patterns associated with serogroups linked to wildlife and rodent reser-

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voirs highlights the need for continued surveillance, rodent control, and careful interpretation of serology in relation to vaccination history.

Keywords: ***Leptospira spp.; Brucella canis; shelter dogs; serological survey; seroprevalence; Croatia*.**

Introduction

Leptospirosis is a globally distributed zoonotic disease that affects a wide range of animal species, including dogs and humans (Levett, 2001; Adler and de la Peña Moctezuma, 2010). The disease is caused by pathogenic spirochetes of the genus *Leptospira*, primarily *Leptospira interrogans* and *Leptospira kirschneri* , which are responsible for most infections in dogs (Sykes et al., 2011). Dogs become infected through direct or indirect contact with contaminated urine, water, or soil, and the bacteria enter the host through mucous membranes or damaged skin (Sykes et al., 2011). Clinical manifestations in dogs vary from subclinical infections to severe disease characterised by pulmonary haemorrhage syndrome, renal and hepatic failure, which can be fatal (Sykes et al., 2011). The diagnosis of leptospirosis is most commonly based on serological testing, particularly the microscopic agglutination test (MAT), which is considered the gold standard (Adler and de la Peña Moctezuma, 2010; Sykes et al., 2011). Antibodies typically develop 5 to 15 days after infection and may persist for extended periods, complicating the interpretation of results (Andre-Fontaine, 2013). Factors such as vaccination, antibiotic treatment, and stage of infection can significantly influence MAT titres, making careful interpretation essential (Fraune et al., 2013; Schuller et al., 2015). Titres may remain detectable for at least one year after natural infection, while vaccine-induced antibodies generally decline within a few months (Barr et al., 2005). Leptospires are maintained in the environment through reservoir hosts, particularly rodents that excrete bacteria in urine and contribute to environmental contamination of soil and water (Yanagihara et al., 2022). Under favourable conditions, leptospires can survive in the environment for weeks to months, facilitating indirect transmission (Yanagihara et al., 2022).

In Croatia, leptospirosis is considered endemic, particularly in the valleys of major rivers such as the Sava and Drava Rivers, and other recognised natural foci with high rodent density, where environmental and ecological conditions favour pathogen persistence and transmission. Previous studies have identified that serovars such as Australis and Grippotyphosa are predominant in both animals and humans, suggesting a strong link between wildlife reservoirs and infection dynamics in domestic species (Turk et al., 2003; Habuš et al., 2008, 2017; Balen Topić et al., 2010). Dogs play an important role in the epidemiology of leptospirosis, both as potential hosts and sentinels of environmental contamination (Ghneim et al., 2007; Schuller et al., 2015). Several studies have identified risk factors for infection, including male sex, urban environments, and exposure to contaminated water sources (Azócar-Aedo and Monti, 2016). In addition, close contact between dogs and humans may contribute to zoonotic transmission, highlighting the importance of canine surveillance within the One Health framework (Jansen et al., 2005). Animal shelters represent a particularly relevant setting for leptospirosis surveillance, as dogs housed in these environments are often exposed to shared spaces, variable hygiene conditions, and potential contact with wildlife or rodent reservoirs (Schuller et al., 2015). These factors may increase the risk of infection and facilitate the circulation of multiple *Leptospira* serovars within the population. Despite this, data on leptospiral exposure in shelter dogs in Croatia remain limited.

Brucella canis is another zoonotic pathogen of concern in canine populations, primarily associated with reproductive disorders such as infertility, abortions, and discospondylitis (Djokić et al., 2023). Transmission occurs mainly through contact with reproductive fluids, aborted materials, urine or semen (Djokić et al., 2023). Although human infections are relatively rare and probably underdiagnosed due to the limitations of diagnostic tests in standard use in human medicine, they can result in chronic and non-specific clinical manifestations, including fever, lymphadenopathy, and splenomegaly (Wallach et al., 2004; Lucero et al., 2010). In recent years, *B. canis* has been increasingly recognised as an emerging pathogen in Europe, particularly associated with dog movement and importation (Escauriaza et al., 2021; Djokić et al., 2023). Since *B. canis* infection is often underdiagnosed due to non-specific clinical signs, serological testing is commonly used for screening, while culture and PCR are considered confirmatory methods for definitive diagnosis (Santos et al., 2021; Djokić et al., 2023). The epidemiology of *B. canis* differs from that of leptospirosis, as infection is more commonly associated

with breeding populations and sexually mature animals. Consequently, the prevalence in shelter dogs is often reported as low or absent, although systematic surveillance remains limited and geographically heterogeneous (Buhmann et al., 2019).

Given the zoonotic importance of both pathogens and the limited data available for shelter dogs in Croatia, this study aimed to: (1) evaluate the prevalence of specific antibodies against *Leptospira* in dogs from two animal shelters, (2) identify the most frequently detected *Leptospira* serogroup/serovar-related MAT reactivity patterns, and (3) assess the seroprevalence of *B. canis* in the same population.

Materials and Methods

Study design and sample collection

This retrospective cross-sectional study was conducted using archived serum samples collected from dogs housed in two animal shelters in Croatia: the Dumovec Shelter for Abandoned Animals of the City of Zagreb (Zagreb County), and the Friends Čakovec Shelter for Abandoned Animals (Međimurje County). The study population consisted of shelter dogs of mixed breeds and unknown prior environmental exposure histories. Sample collection was performed as part of routine shelter veterinary care and preventive health monitoring in dogs without visible clinical signs of disease, rather than as part of diagnostic work-up in dogs with clinically suspected leptospirosis or brucellosis. A total of 375 dogs met the inclusion criteria and were included in the analysis. Dogs were eligible for inclusion if a serum sample of sufficient volume was available for serological testing and if basic metadata were available, including shelter of origin and sex; age information was included when available. Dogs were categorised into four age groups for analysis: <1 year, 1–3 years, 4–7 years, and ≥8 years. Of the 375 dogs included in the study, 197 were males and 178 were females. Vaccination status and the time elapsed since the last leptospirosis vaccination were recorded where available. Because shelter dogs may have incomplete vaccination histories, vaccination-adjusted analyses were performed only for animals with reliable information on the timing of leptospirosis vaccination. Blood samples were collected by venipuncture into serum tubes as part of routine veterinary procedures. After clotting and centrifugation, serum was separated and stored under controlled conditions until analysis. Each dog contributed one serum sample for leptospirosis MAT testing and *B. canis* antibody screening.

Serological Testing for Leptospirosis

Serological testing for leptospirosis was performed using the microscopic agglutination test (MAT) on serum samples, following the recommendations of the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2018). MAT was performed in the Laboratory for Bacterial Zoonoses and Molecular Diagnostics of Bacterial Diseases (Croatian Veterinary Institute), using live antigens maintained as pure cultures. The MAT antigen panel included ten pathogenic serovars representing serogroups known to circulate regionally and/or to be clinically relevant in dogs and public health. The following serovars/serogroups were included as antigens: Australis (serovar Bratislava), Ballum (serovar Ballum), Bataviae (serovar Bataviae), Canicola (serovar Canicola), Grippotyphosa (serovar Grippotyphosa), Icterohaemorrhagiae (serovar Copenhageni), Pomona (serovar Pomona), Sejroe (serovar Hardjo and Saxkoebing), and Tarassovi (serovar Tarassovi). The inclusion of these serovars was intended to capture both classical canine serovars (e.g., Canicola and Icterohaemorrhagiae) and serovars increasingly associated with canine exposure in Europe and Croatia, including Australis and Grippotyphosa (Adler and de la Peña Moctezuma, 2010; Sykes et al., 2011; Habuš et al., 2017). Serum samples were initially screened at a dilution of 1:50 and then serially diluted in two-fold steps to determine endpoint titers. The MAT titre was defined as the highest serum dilution showing ≥50% agglutination of leptospires when compared with the negative control. A titre of ≥1:50 was considered positive. Because MAT may show substantial cross-reactivity between serogroups, results were interpreted cautiously as reactivity to the included MAT antigens rather than as definitive identification of the infecting serovar. For descriptive purposes, the highest reactive titre was recorded for each antigen, and when multiple antigens showed similar reactivity, this was recorded as reactivity to multiple serovars/serogroups, consistent with the known interpretative limitations of MAT (Adler and de la Peña Moctezuma, 2010; Sykes et al., 2011; Fraune et al., 2013; Schuller et al., 2015). Because MAT detects agglutinating antibodies that may arise both from natural infection and vaccination, interpretation is influenced by vaccination status and time since vaccination (Barr et al., 2005; Fraune et al., 2013; Schuller et al., 2015). To explore the possible influence of vaccination on MAT results, a vaccination-related sensitivity analysis was performed for dogs with known recent vaccination. Specifically, titres to antigens representing the vaccine-included serovars Icterohaemorrhagiae and Canicola detected within 0–20 weeks (0–5 months) after vaccination were considered potentially vaccine-associated and were therefore excluded from serovar-specific analyses for these two serovars. This approach was based on the fact that bivalent canine leptospirosis

vaccines used in Croatia, such as Vanguard 7 (European Medicines Agency, 2024), include the serovars Canicola and Icterohaemorrhagiae, which may induce detectable post-vaccinal MAT titres. This 0–20-week window was selected as a conservative period based on published evidence showing that vaccine-associated MAT titres may persist for several weeks to months and can remain detectable beyond 16 weeks depending on the serovar and vaccine formulation (Barr et al., 2005; Andre-Fontaine, 2013). The sensitivity analysis was intended to minimise false attribution of recent post-vaccinal titers to natural exposure in a shelter population where clinical follow-up is limited and vaccination histories may be incomplete (Barr et al., 2005; Fraune et al., 2013; Schuller et al., 2015; Sykes et al., 2023). However, because complete product-level vaccination histories were not consistently available for all dogs, and both bivalent and tetravalent canine leptospirosis vaccines are available in Croatia, this analysis was interpreted cautiously. It was intended only to explore the possible contribution of recent vaccination to MAT reactivity and should not be interpreted as a definitive correction for vaccine-induced antibodies or as a basis for recalculating overall seroprevalence.

Serological Testing for *Brucella canis*

Serological testing for *B. canis* was performed on all serum samples (n = 375) using a rapid immunochromatographic assay (Anigen Rapid C. Brucella Ab Test Kit, BioNote, Gyeonggi, Republic of Korea), according to the manufacturer's instructions. This lateral flow assay is designed for the qualitative detection of antibodies against *B. canis* in canine serum. Results were interpreted visually, where the presence of both control and test lines indicated a positive result, while the presence of only the control line was considered negative. Internal controls included in the test ensured the validity of each assay. Although this method is suitable for rapid screening, it should be noted that immunochromatographic assays may have lower sensitivity compared to culture or molecular methods such as PCR, particularly in early or chronic stages of infection.

Statistical analysis

Statistical analyses were performed using Stata version 13.1 (StataCorp, College Station, TX, USA). The chi-square test was used to assess associations between categorical variables, including sex and serovar distribution, as well as differences between shelters. Spearman's rank correlation coefficient was used to evaluate the relationship between age and the number of detected serovars. Differences in antibody titres among serovars were assessed using the Kruskal-Wallis test. When appropriate, pairwise comparisons were conducted using the Mann-Whitney U test with Bonferroni correction for multiple testing. A p-value of <0.05 was considered statistically significant.

Results

Serological Testing for Leptospirosis

Of the total collected specimens, 375 met the inclusion criteria. For 128 of 375 dogs (34.1%), the test was positive with a titer ≥50 to at least one of the serovars examined. Of the 115 dogs tested from the Dumovec shelter, 34 (29.6%) were seropositive, while 94 of 260 dogs (36.1%) tested from the Čakovec shelter were positive.

Table 1. Frequency of MAT reactivity to *Leptospira* antigens among seropositive dogs (n = 128) from two Croatian animal shelters (Dumovec and Čakovec)

Serovar	Occurrences (%)		
	Dumovec Shelter (n=34 positive dogs)	Čakovec Shelter (n=94 positive dogs)	Total number (n=128 positive dogs)
Icterohaemorrhagiae	19 (55.9%)	63 (64.3%)	82 (64.1%)
Australis	16 (47.1%)	61 (62.2%)	77 (60.2%)
Canicola	13 (38.2%)	43 (43.9%)	56 (43.7%)
Pomona	9 (26.5%)	40 (40.8%)	49 (38.3%)
Grippotyphosa	5 (14.7%)	26 (26.5%)	31 (24.2%)
Sejroe	2 (5.9%)	10 (10.2%)	12 (9.4%)
Saxkoebing	1 (2.9%)	6 (6.1%)	7 (5.5%)
Ballum	0 (0.0%)	2 (2.0%)	2 (1.6%)
Hardjo	0 (0.0%)	1 (1.0%)	1 (0.8%)

Table 2. Distribution of MAT reactivity to Leptospira antigens among seropositive dogs by sex (males n = 74, females n = 54; total n = 128). Individual dogs could be seropositive to more than one serovar.

Serovar	Female (F)	Male (M)
Icterohaemorrhagiae	39	43
Australis	31	46
Canicola	16	23
Pomona	22	30
Grippotyphosa	18	27
Sejroe	7	5
Saxkoebing	4	3
Ballum	1	1
Hardjo	0	1

Table 3. Distribution of MAT reactivity to Leptospira antigens by age group

Age Group	ICT	AUS	CAN	POM	GRI	SEJ	SAX	BAL	HAR
<1 year	0	0	0	0	0	0	0	0	0
1-3 years	22	21	9	14	11	4	3	0	0
4-7 years	39	33	14	20	23	6	2	0	0
8+ years	7	7	7	4	3	1	1	1	1

ICT – Icterohaemorrhagiae; AUS - Australis; CAN – Canicola; POM – Pomona; GRI – Grippotyphosa; SEJ – Sejroe; SAX – Saxkoebing; BAL – Ballum; HAR – Hardjo

Of the 375 tested dogs, 197 were males and 178 were females. Seropositivity was detected in 74/197 males (37.6%) and 54/178 females (30.3%), with no statistically significant difference in overall seroprevalence between sexes ($\chi^2 = 2.17$, $p = 0.141$).

The most frequent MAT reactivity across all dogs tested was directed against the antigen representing Icterohaemorrhagiae, observed in 82/128 seropositive dogs (64.1%). Other frequent MAT reactions were directed against antigens representing Australis (60.2%), Canicola (43.7%), Pomona (38.3%), and Grippotyphosa (/128; 24.2%). Less frequent reactions were recorded against antigens representing Sejroe (9.4%), Saxkoebing (5.5%), Ballum (1.6%), and Hardjo (0.8%) (Table 1). Since individual dogs may react to multiple antigens in the MAT, these findings are presented as reactivity patterns rather than definitive identification of infecting serovars.

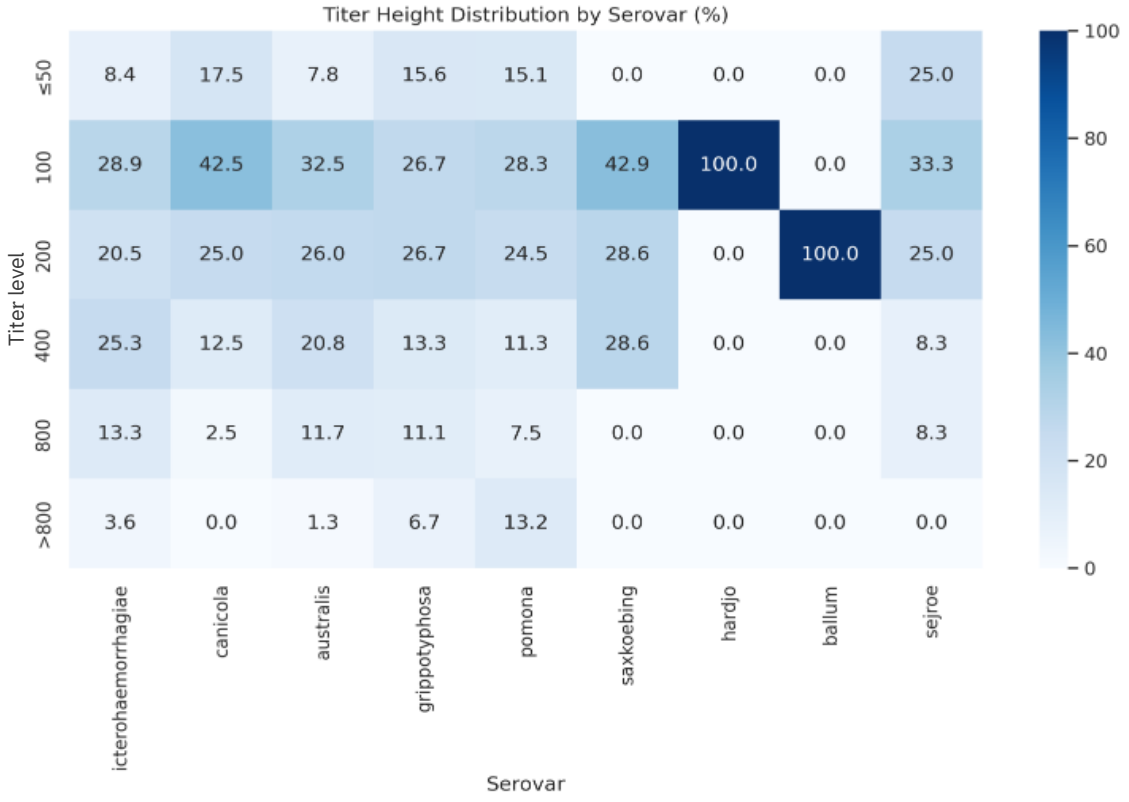
Serovar profiles were comparable between the Dumovec and Čakovec shelters, with no marked differences in the most frequently detected serovars. Because individual dogs could react to multiple serovars in MAT, comparisons of serovar profiles between groups were interpreted descriptively.

When analysing MAT reactivity patterns by sex, males showed slightly higher positivity rates for both Icterohaemorrhagiae (43 vs. 39) and Australis (46 vs. 31). In contrast, females had a marginally higher prevalence of Sejroe (7 vs. 5) (Table 2). MAT reactivity profiles were similar between male and female dogs, with no apparent differences in the most frequently detected serovars.

Age-related distribution of MAT reactivity revealed that dogs aged 4 to 7 years had the highest seropositivity rates, particularly for Icterohaemorrhagiae (39 cases), followed by Australis (33 cases), and Grippotyphosa (23 cases). Dogs aged 1 to 3 years also showed significant levels of exposure, though generally lower than those in the 4–7 year age group. The oldest age group (8+ years) had the fewest cases across all serovars, and notably, no seropositivity was observed in any dogs younger than one year (Table 3).

The correlation coefficient was 0.047 with a p-value of 0.629, indicating no significant correlation between age and serovar count. Furthermore, when individual MAT reactivity patterns were analysed in

Figure 1. Heatmap showing the distribution of MAT antibody titres across MAT-reactive antigens. Darker shades indicate a higher proportion of positive samples at a given titer level.



relation to age, none showed a statistically significant association ($p > 0.05$ for all serovars). The serovar Canicola approached significance ($p = 0.072$), but remained above the conventional threshold.

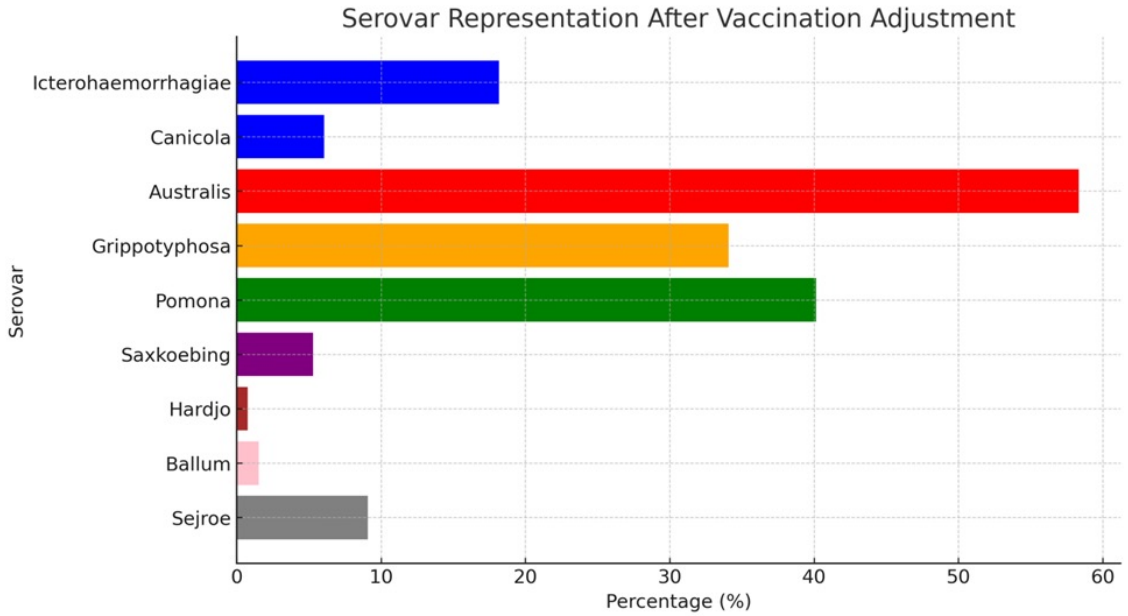
Antibody titres were predominantly low to moderate across most MAT-reactive serovars, with titres of 1:100 and 1:200 being the most frequently observed. For Icterohaemorrhagiae, Canicola, Australis, Grippotyphosa, and Pomona, the most common titres were generally 1:100 or 1:200, while higher titres were less frequent. Among these, Pomona showed the highest upper-end reactivity, with titres reaching 1:6400, whereas the highest titres for Icterohaemorrhagiae, Australis, and Grippotyphosa reached 1:3200, 1:1600, and 1:1600, respectively. Reactivity to Saxkoebing, Hardjo, and Ballum was rare and limited to a small number of dogs with low to moderate titres. Overall, the titre distribution was dominated by low and intermediate values, while very high titres were uncommon (Figure 1).

The Kruskal-Wallis H-test resulted in a test statistic of 12.22 with a corresponding p-value of 0.142. Since the p-value exceeds the conventional significance threshold of 0.05, no statistically significant differences in antibody titre values were observed among the serovars. While some serovars, such as Pomona, exhibited higher median titres, the overall variation across serovars was not sufficient to reach statistical significance. Further analysis using pairwise Mann-Whitney U tests with Bonferroni correction for multiple comparisons confirmed these findings, as none of the pairwise comparisons yielded statistically significant results (all p-values > 0.05).

In the vaccination-related sensitivity analysis, when potentially vaccine-associated reactivity to antigens representing Icterohaemorrhagiae and Canicola in recently vaccinated dogs was examined separately, the most frequent MAT reactivity patterns remained those directed against antigens representing Australis (58.33%), Pomona (40.15%), and Grippotyphosa (34.09%). Lower frequencies were observed for reactivity to antigens representing Icterohaemorrhagiae (18.18%), Canicola (6.06%), Sejroe (9.09%), Saxkoebing (5.30%), Ballum (1.52%), and Hardjo (0.76%) (Figure 2). However, this analysis should be interpreted cautiously because complete vaccine-product information was not consistently available and tetravalent vaccines may also influence MAT reactivity patterns. No statistically significant differences in MAT reactivity patterns across age groups were observed in this sensitivity analysis (all $p > 0.05$).

In the vaccination-related sensitivity analysis, low to moderate titres remained the most common

Figure 2. Horizontal bar chart showing the frequency of MAT reactivity to *Leptospira* antigens in the vaccination-related sensitivity analysis, after separate evaluation of potentially vaccine-associated reactivity to antigens representing Icterohaemorrhagiae and Canicola in recently vaccinated dogs



across MAT-reactive antigens, with 1:100 and 1:200 being the predominant titres. Higher titres were uncommon and were observed mainly in reactions to antigens representing Pomona, Icterohaemorrhagiae, Australis, and Grippotyphosa (Table 4).

Serological Testing for *Brucella canis*

All 375 serum samples were tested for the presence of *B. canis* antibodies using a chromatographic immunoassay. No samples tested positive, indicating a seroprevalence of 0% in the studied population of shelter dogs.

Discussion

This study demonstrated a high seroprevalence of *Leptospira* spp. antibodies among dogs from two Croatian animal shelters, indicating substantial environmental exposure to pathogenic leptospires in this population. These findings are consistent with the endemicity of leptospirosis in Croatia, particularly in continental areas and river valleys where ecological conditions favour pathogen persistence in wildlife reservoirs and the environment (Borčić et al., 1982, 1983; Zaharija et al., 1982; Pappas et al., 2008; Balen Topić et al., 2010; Habuš et al., 2017). In this context, shelter dogs may serve as useful sentinels of local environmental contamination within a One Health framework (Ghneim et al., 2007; Schuller et al., 2015; Sykes et al., 2023).

Before vaccination adjustment, the most frequent MAT reactions were directed against antigens representing Icterohaemorrhagiae, Australis, Canicola, Pomona and Grippotyphosa. This broadly agrees with previous European and Croatian studies showing that canine exposure is no longer limited to the classical serovars Canicola and Icterohaemorrhagiae, but increasingly includes Australis and Grippotyphosa-related infections (Geisen et al., 2007; Mastrorilli et al., 2007; Ellis, 2010; Tagliabue et al., 2016; Schuller et al., 2015; Habuš et al., 2017). After cautious adjustment for recent vaccination, MAT reactivity against antigens representing Australis, Pomona and Grippotyphosa remained the most frequent pattern, suggesting that part of the initial reactivity to Icterohaemorrhagiae and Canicola was vaccine-associated. This is biologically plausible because these serovars are included in bivalent canine leptospirosis vaccines used in Croatia, and vaccine-induced MAT titres may persist for weeks to months after vaccination (Barr et al., 2005; Andre-Fontaine, 2013; Schuller et al., 2015; Sykes et al., 2023). However, because tetravalent leptospirosis vaccines are also available in Croatia and complete vaccine-product information was not consistently available, this finding should be interpreted with caution.

The predominance of MAT reactivity to antigens representing Australis, Pomona, and Grippotyphosa is epidemiologically relevant because these serogroup-related patterns have also been associated with wildlife reservoirs in Croatia. Previous studies linked Icterohaemorrhagiae mainly to brown rats, Sejroe to house mice, Grippotyphosa to field voles, and Pomona to striped field mice,

Table 4. Distribution of titre levels for MAT-reactive antigens after the vaccination-related sensitivity analysis

Titer Level	ICT	CAN	AUS	GRI	POM	SAX	HAR	BAL	SEJ
1:50	13.6%	12.5%	7.8%	15.6%	13.5%	0%	0%	0%	25.0%
1:100	13.6%	50.0%	32.5%	26.7%	28.8%	42.9%	100%	0%	33.3%
1:200	36.4%	25.0%	26.0%	26.7%	25.0%	28.6%	0%	100%	25.0%
1:400	22.7%	12.5%	20.8%	13.3%	11.5%	28.6%	0%	0%	8.3%
1:800	9.1%	0%	11.7%	11.1%	7.7%	0%	0%	0%	8.3%
1:1600	0%	0%	1.3%	6.7%	7.7%	0%	0%	0%	0%
>1:1600	4.6%	0%	0%	0%	5.8%	0%	0%	0%	0%

ICT-Icterohaemorrhagiae, CAN-Canicola, AUS-Australis, GRI-Grippotyphosa, POM-Pomona, SAX-Saxkoebing, HAR-Hardjo, BAL-Ballum, SEJ-Sejroe

while Australis has been isolated from several species of small wild rodents (Borčić et al., 1982, 1983; Turk et al., 2003; Habuš et al., 2008). These findings support the interpretation that the dogs in this study were primarily exposed indirectly through contaminated environments shared with synanthropic and wild rodents, which is particularly relevant in shelter settings (Schuller et al., 2015; Yanagihara et al., 2022).

These MAT reactivity patterns are also relevant from a public health perspective. In Croatia, Australis and Grippotyphosa have repeatedly been recognised among important probable infecting serovars in both humans and animals, while human leptospirosis in several other European countries is more often dominated by Icterohaemorrhagiae (Ciceroni et al., 2000; Jansen et al., 2005; Milas et al., 2005; Habuš et al., 2008, 2017). Although the dogs in the present study were not sampled as clinically suspected cases and no direct transmission link to humans can be inferred, the overlap between canine and human epidemiologically relevant serovars supports the value of shelter dogs as sentinels of zoonotic risk. In addition, serovars from the Icterohaemorrhagiae serogroup remain classically associated with severe human leptospirosis, including Weil's disease (Levett, 2001; Adler and de la Peña Moctezuma, 2010).

Most MAT titres were in the low to moderate range, which is generally more consistent with previous exposure than with acute infection, particularly in the absence of clinical data (Adler and de la Peña Moctezuma, 2010; Schuller et al., 2015; Sykes et al., 2023). However, some dogs showed high titres, especially to Pomona and Icterohaemorrhagiae, which may indicate more recent or more intense exposure (Fraune et al., 2013; Sykes et al., 2023). No significant associations were found between seropositivity and sex, shelter of origin, or age, suggesting that exposure risk in this population was relatively widespread and driven more by shared environmental conditions than by host demographic factors (Ghneim et al., 2007; Azócar-Aedo & Monti, 2016). Because this was a retrospective cross-sectional survey, seasonal variation could not be assessed directly, although the high overall seroprevalence is consistent with persistent environmental exposure in Croatia (Sykes et al., 2007; Balen Topić et al., 2010; Habuš et al., 2017).

In contrast, no serological evidence of *B. canis* infection was detected in any tested dog, suggesting that the burden of this pathogen in the investigated shelters is very low. Similar low or negative seroprevalence has been reported in non-breeding canine populations, whereas higher prevalence is more often associated with breeding kennels, imported dogs, or uncontrolled reproduction (Buhmann et al., 2019; Santos et al., 2021; Djokić et al., 2023). Nevertheless, these findings should be interpreted cautiously because serological screening methods may be less sensitive in early or chronic infection, and active infection cannot be excluded without culture or molecular testing (Santos et al., 2021; Djokić et al., 2023; Barros et al., 2024). Continued surveillance therefore remains justified, particularly given increasing concern regarding *B. canis* in Europe (Buhmann et al., 2019; Escauriaza et al., 2021; Djokić et al., 2023).

This study has several limitations. The retrospective cross-sectional design does not allow for determination of infection timing, clinical relevance, or urinary shedding. Although the dogs had no visible clinical signs of disease at the time of sampling and were not sampled as clinically suspected cases, subclinical or early infection could not be excluded. MAT cannot fully distinguish between infection-induced and vaccine-associated antibodies, and serological cross-reactivity re-

mains an inherent limitation of the method (Adler and de la Peña Moctezuma, 2010; Andre-Fontaine, 2013; Schuller et al., 2015; Sykes et al., 2023). In addition, the vaccination-related sensitivity analysis should be interpreted cautiously because complete vaccine-product information was not consistently available for all dogs. Molecular confirmation of *Leptospira* or *B. canis* infection was not performed, and the study was limited to two shelters, which may reduce generalisability. Despite these limitations, the study provides valuable contemporary data on exposure to two important zoonotic pathogens in Croatian shelter dogs and supports continued surveillance, rodent and environmental control, and careful interpretation of serological findings in relation to vaccination history (Schuller et al., 2015; Buhmann et al., 2019; Djokić et al., 2023; Sykes et al., 2023).

Conclusion

In conclusion, shelter dogs in the investigated Croatian shelters showed a high seroprevalence of exposure to pathogenic *Leptospira* spp., with predominant MAT reactivity to antigens representing the Australis, Pomona and Grippotyphosa serogroups, whereas no serological evidence of *B. canis* infection was detected. These findings support the role of shelter dogs as sentinels of environmental exposure to leptospires and underline the importance of continued surveillance, improved environmental and rodent control, and careful interpretation of serological results in relation to vaccination history. The observed epidemiological pattern further highlights the relevance of a One Health approach integrating animal, environmental, and public health perspectives.

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> Retrospektivno serološko istraživanje izloženosti pasa iz hrvatskih skloništa bakterijama roda *Leptospira* i vrsti *Brucella canis*

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U ovom retrospektivnom presječnom istraživanju ispitana je izloženost pasa iz dva skloništa u Hrvatskoj bakterijama roda *Leptospira* i vrsti *Brucella canis*. Analizirani su pohranjeni serumi 375 pasa iz skloništa Dumovec (Zagrebačka županija) i skloništa Prijatelji Čakovec (Međimurska županija). Protutijela na Leptospira spp. određena su mikroskopskim aglutinacijskim testom (MAT)

primjenom panela od 10 patogenih serovara, dok su protutijela na *B. canis* pretražena brzim imunokromatografskim testom. Statistički su analizirane povezanosti sa skloništem, spolom, dobi, raspodjelom serovara i titrovima protutijela. Protutijela na najmanje jedan serovar *Leptospira* spp. utvrđena su u 128/375 pasa (34,1%). Seropozitivnost je iznosila 29,6% u Dumovcu i 36,1% u Čakovcu, bez značajne razlike između skloništa. Nije utvrđena statistički značajna povezanost ukupne seropozitivnosti sa spolom ni dobi. Prije korekcije na nedavno cijepljenje najčešće su utvrđeni serovari Icterohaemorrhagiae (64,1%), Australis (60,2%), Canicola (43,7%), Pomona (38,3%) i Grippotyphosa (24,2%). Nakon isključenja mogućih postvakcinalnih titrova za Icterohaemorrhagiae i Canicola u nedavno cijepljenih pasa, najzastupljeniji su bili Australis, Pomona i Grippotyphosa. Većina titrova bila je niska do umjerena, što više upućuje na prethodnu izloženost nego na akutnu infekciju. Protutijela na *Brucella canis* nisu dokazana ni u jednog od 375 pasa. Rezultati upućuju na značajnu okolišnu izloženost pasa iz skloništa patogenim leptospirama u Hrvatskoj te potvrđuju njihovu vrijednost kao sentinel-populacije za procjenu zoonotskog rizika. Nalaz serovara povezanih s glodavcima i divljim životinjama naglašava potrebu za trajnim nadzorom, kontrolom glodavaca i opreznim tumačenjem seroloških nalaza u odnosu na cijepljenje.

Ključne riječi: *Leptospira spp.; Brucella canis; psi iz skloništa; serološko istraživanje; seroprevalencija; Hrvatska*.