Identification of the most effective serovars to be included in the MAT antigen panel to optimize the serodiagnosis of Leptospira infection in Northern Italy

Short Communication / Brief Report

Identification of the most effective serovars to be included in the MAT antigen panel to optimize the serodiagnosis of *Leptospira* infection in Northern Italy

Andrea Balboni [1], Mario D'Incau [2], Silvia Zamagni [3], Laura Lucchese [4], Elisa Mazzotta [4], Silvia Marchione [4], Mara Battilani [1], Alda Natale [4]*

[1] Department of Veterinary Medical Sciences, *Alma Mater Studiorum*-University of Bologna, 40064 Ozzano Emilia, Bologna, Italy.

[2] Italian Reference Centre for Animal Leptospirosis, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini", 25121 Brescia, Italy.

[3] Veterinary practitioner at the Centro Veterinario Romagnolo, 47853 Coriano (RN), Italy.

[4] Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università, 35020 Legnaro, Italy.

* Corresponding author:

Alda Natale

Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università, 35020 Legnaro, Italy.

E-mail address: anatale@izsvenezie.it

ORCID:

Andrea Balboni: https://orcid.org/0000-0002-8049-6645

Abstract

The microscopic agglutination test (MAT) assay is adopted as a world reference test for the serodiagnosis of leptospirosis in humans and animals. The incapability of MAT to detect antibodies against leptospiral serogroups not included in the assay antigen panel is one of the main limitations of this test and serodiagnostic antigens should be periodically updated with locally circulating serovars in order to optimise its performance. The aim of this study was to determine the need to implement the antigen panel currently adopted in Northern Italy for the diagnosis of *Leptospira* infection in dogs. For this purpose, a group of 288 dogs with and without clinical signs potentially associated with *Leptospira* infection or increased C-reactive protein (CRP) serum concentration, sampled in 2013-2016 in Northern Italy, were tested by MAT comparing the results obtained with a nine antigens panel (Australis-Bratislava, Ballum-Ballum, Canicola-Canicola, Grippotyphosa-Grippotyphosa, Icterohaemorrhagiae-Copenhageni, Icterohaemorrhagiae-Icterohaemorrhagiae, Sejroe-Hardjo, Pomona-Pomona and Tarassovi-Tarassovi serovars) routinely adopted and a panel expanded to 27 antigens. In general, the antigen panel currently adopted in Northern Italy for the routine MAT assay resulted adequate for the diagnosis of *Leptospira* infection in dogs. The main exception concerns the Sejroe serogroup, with the Saxkoebing and Sejroe serovars that were more effective than Hardjo for diagnosis in dogs and whose inclusion in the antigen panel is recommended. Among other antigens evaluated in this study, Cynopteri serovar was detected with high frequency but its pathogenic role in dogs and as public health threat deserve further investigation.

Keywords: antigen, diagnosis, dog, *Leptospira*, microscopic agglutination test, serology

Introduction

Leptospirosis is a worldwide zoonosis affecting numerous wild and domestic mammalian species (Bharti et al., 2003), sustained by pathogenic Gram-negative and highly motile spirochete bacteria of the genus *Leptospira*. Some host species act as reservoirs, representing the natural source of infection and of environmental contamination (Gomard et al., 2021; Levett, 2001). Reservoir hosts are persistently infected, normally with no clinical signs, and shed bacteria through their urine even lifelong (Schuller et al., 2015). Differently, incidental hosts can develop acute and severe disease (Levett, 2001; Schuller et al., 2015). The dog is usually an incidental host, showing a wide range of clinical manifestations, from subclinical to severe (Sykes et al., 2011; Schuller et al., 2015), but can represent an important sentinel species as well as a potential reservoir host for some serovars (Balboni et al., 2022).

Leptospira spp. are classified in hundreds of serovars due to variable epitopes in the lipopolysaccharide (LPS) structure; furthermore, different serovars are grouped into serogroups (Faine et al., 1999; Ko et al., 2009). Most of the known serovars have close relationships with specific reservoir hosts and the epidemiology of canine leptospirosis can vary by geographic area and over time, in relation to the spread of maintenance hosts and vaccination (Bharti et al., 2003; Schuller et al., 2015; Sykes et al., 2010). In Europe, dogs are apparently more exposed to Icterohaemorrhagiae, Grippotyphosa, Australis, Canicola and Sejroe serogroups (Ellis, 2010), but many other serogroups such as Autumnalis, Ballum, Bataviae, Cynopteri, Pomona, Pyrogenes and Tarassovi were reported worldwide in dogs (Costa et al., 2022; Pinto et al., 2017; Sykes et al., 2011), also in association with clinical manifestations, and reservoirs of some serovars still remain unknown.

The vaccination evokes a serovar-specific and partially serogroup-specific immune protection (Bouvet et al., 2020; Klaasen et al., 2022). To date, trivalent or tetravalent vaccines containing antigens from up to four different serovars belonging to Canicola, Icterohaemorrhagiae, Australis,

and Grippotyphosa serogroups are available in Europe and Italy (Ellis, 2010; Klaasen et al., 2014; Schuller et al., 2015).

Diagnosis of leptospirosis should be multifaceted and not rely on any one single test (Sykes et al., 2022). To achieve a definitive diagnosis many factors should be considered, such as potential exposure, clinical presentation and laboratory values, and the results of multiple direct or indirect diagnostic approaches must be evaluated (Sykes et al., 2022). The microscopic agglutination test (MAT) involves incubation of serial dilutions of patient sera with a panel of live leptospiral organisms as antigens and reading the resulting agglutination under a darkfield microscope (Sykes et al., 2022). MAT is a serogroup rather than a serovar-specific test (Levett, 2001); nevertheless, different responses are detectable between serovars belonging to the same serogroup. Although MAT is subjected to a number of limitations (Barr et al., 2005; Kohn et al., 2010; Martin et al., 2014; Schuller et al., 2015; Sykes et al., 2010; Sykes et al., 2022), it is still being adopted as a world reference test for the serodiagnosis of leptospirosis in humans and animals (World Organisation for Animal Health, 2022). The incapability of MAT to detect antibodies against leptospiral serogroups not included in the assay antigen panel is one of the main limitations of this test. For this reason, live antigen panels should include locally circulating serovars and serodiagnostic antigens should be periodically updated as new strains emerge in order to optimise its performance (Sykes et al., 2022). In this study, a group of dogs showing clinical signs potentially associated with *Leptospira* infection or increased C-reactive protein (CRP) serum concentration and a group of apparently healthy dogs were tested by MAT comparing the results obtained with a nine antigens panel routinely used in Northern Italy and a panel expanded to 27 antigens, in order to determine the need to implement the antigen panel currently adopted in Northern Italy for the diagnosis of *Leptospira* infection in dogs.

Materials and Methods

Study design, population and sampling

This retrospective study was carried out at the Istituto Zooprofilattico Sperimentale delle Venezie, (IZSVe, Legnaro, Padova, Italy) and the Italian Reference Centre for Animal Leptospirosis (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, IZSLER, Brescia, Italy). In the study, serum samples from dogs were selected and sent to the IZSVe laboratory for diagnostic purposes, and the implementation of the research study was supported by funding from the RC IZS VE 16/12, from August 2013 to July 2016. The study population was composed by owned dogs showing clinical signs potentially associated with *Leptospira* infection or increased CRP serum concentration (Ceron et al., 2005; Schuller et al., 2015) sampled by veterinary practitioners, apparently healthy kennel dogs undergoing neutering surgery sampled to perform pre-operative profile tests and apparently healthy blood donor dogs sampled to perform pre-donation screening tests. No dogs were sampled exclusively for the purposes of this study. Only samples taken for diagnostic purposes following owner or legal manager of the kennel consent were used.

Blood sampling was carried out by venepuncture and serum samples were stored at -20 °C until analysis. Signalment data and vaccination status were retrieved from medical records. Vaccination status was compared to international guidelines for the vaccination of dogs (Day et al., 2016).

All dogs included in the study were tested by MAT both with a nine antigens panel routinely adopted in Northern Italy, in line with the eight antigen panel fixed at national level by the National Reference Laboratory for Leptospirosis to which the Icterohaemorrhagiae serovar was added (Tagliabue, 2016), and a panel expanded to 27 antigens selected on the basis of epidemiological data from Europe and Mediterranean basin (Arent et al., 2013; Benkirane et al., 2016; Goris et al., 2013; Mayer-Scholl et al., 2013). The results obtained with the two panels were compared to detect seroreactions against antigens not included in the routine test.

Microscopic agglutination test (MAT)

Dog serum samples were tested for antibodies against *Leptospira* using the MAT following the World Organisation for Animal Health (WOAH) method (Chap 3.1.12) (World Organisation for

Animal Health, 2022). In the assay routinely adopted in Northern Italy, the antigen panel included eight serogroups and nine serovars (**Table 1**). In the expanded assay, the antigen panel included 20 serogroups and 27 serovars (**Table 1**), including those of the routine assay. Serum samples were pre-tested at the final dilution of 1:100. Serum with 50% agglutination were retested to determine an endpoint using dilutions of serum beginning at 1:100 through to 1:6400. Serum samples with the widely accepted minimum significant titre of 1:100 (reciprocal of the final dilution of serum with 50% agglutination) were assessed positive. Positive antibody titres ≥ 1:800 against at least one *Leptospira* serogroup were recognised as of potential infectious origin, excluding most vaccine responses.

Addition to the antigen panel has been suggested for serovars that showed a titre ≥ 1:100 in dogs tested negative by the routine MAT assay or a titre equal to or higher than that obtained for serovars used routinely in Northern Italy.

Statistical analysis

The data were evaluated using standard descriptive statistics and reported as median and range. Categorical data were analysed using the Chi-squared test. Statistical significance was set at $P<0.05$. Not available data was excluded to statistical analysis. Statistical analysis was carried out using commercially available software (MedCalc Statistical Software version 16.8.4).

Results

Study population

During the study period, 288 dogs were included in the study: 217/288 (75.3%) were owned dogs showing clinical signs potentially associated with *Leptospira* infection or increased CRP serum concentration and 71/288 (24.7%) dogs were apparently healthy kennel dogs (N: 20) or blood donor dogs (N: 51). Among the study population, 108/288 (37.5%) dogs were males and 94/288 (32.6%) were females, for the remaining 86/288 (29.9%) dogs this data was not available. The median age

147 of dogs (N: 155) was five years (range 1 month – 16 years), whereas, 133/288 (46.2%) were
148 purebred, 60/288 (20.8%) were mixed breed and for the remaining 95/288 (33%) this data was not
149 available. Ninety-four out of 288 (32.6%) dogs were regularly vaccinated against leptospirosis with
150 bivalent (N: 84; Canicola and Icterohaemorrhagiae serogroups) or tetravalent (N: 10; Canicola,
151 Icterohaemorrhagiae, Australis and Grippotyphosa serogroups) vaccines, 59/288 (20.5%) were not
152 or not regularly vaccinated and for the remaining 135/288 (46.9%) this data was not available
153 (**Table 2**).
154
155 ***Routine and expanded MAT assays results***
156 The sera of 128/288 (44.4%) dogs included in the study were tested positive with a cut-off ≥ 1:100
157 to antibodies against at least one of the pathogenic *Leptospira* serovars included in the nine antigens
158 panel of the routine MAT assay (**Table 3**). Adopting a cut-off ≥ 1:800, the number of dogs tested
159 positive was 46/288 (16%).
160 The sera of 141/288 (49%) dogs included in the study were tested positive with a cut-off ≥ 1:100 to
161 antibodies against at least one of the *Leptospira* serovars included in the 27 antigens panel of the
162 expanded MAT assay (**Table 3**). Adopting a cut-off ≥ 1:800, the number of dogs tested positive was
163 52/288 (18.1%).
164 The majority of the seropositive dogs reported multiple titres against different serovars and
165 serogroups. The frequency of detection of the different serovars is summarized in **Table 1**.
166 Considering a cut-off ≥ 1:100, five of the 10 most frequently detected serovars were not included in
167 the routine MAT assay (Australis-Jalna, Autumnalis-Autumnalis, Cynopteri-Cynopteri, Lyme-
168 Lyme and Pyrogenes-Pyrogenes), whereas, considering a cut-off ≥ 1:800, four of the 10 most
169 frequently detected serovars were not included in the routine MAT assay (Australis-Jalna,
170 Autumnalis-Autumnalis, Cynopteri-Cynopteri, and Pomona-Mozdok). The Australis-Bratislava,
171 Canicola-Canicola, Grippotyphosa-Grippotyphosa, Icterohaemorrhagiae-Copenhageni,
172 Icterohaemorrhagiae- Icterohaemorrhagiae and Pomona-Pomona serovars, included in the routine

MAT assay, were among the most frequently detected using both cut-offs **Table 1**. Antibodies against Celledoni-Celledoni, Mini-Mini, Shermani-Shermani and Tarassovi-Tarassovi serovars were not found in this study, whereas Bataviae-Bataviae and Hebdomadis-Hebdomadis serovars were sporadically detected (in one and two dogs, respectively) with low titres (< 1:800, **Table 1**). Using the MAT assay with the expanded 27 antigens panel, 13/288 (4.5%) dogs were tested seropositive only against at least one of the 18 *Leptospira* antigens not included in the routine MAT assay (**Table 3**). Of these dogs, 11 showed antibody titre values < 1:800 against Cynopteri-Cynopteri (N: 1), Hurstbridge-Hurstbridge (N: 3), Lyme-Lyme (N: 5) and Pyrogenes-Pyrogenes (N: 1) serovars, and two showed antibody titre value ≥ 1:800 against Sejroe-Saxkoebing serovar (1:800 and 1:3200, respectively). The dog tested positive to Sejroe-Saxkoebing serovar with titre 1:3200 also showed seropositivity against Lyme-Lyme (1:200) and Sejroe-Sejroe (1:100) serovars. In addition, four dogs had MAT titres ≥ 1:800 against at least one of the 18 *Leptospira* antigens not included in the routine assay (Cynopteri-Cynopteri N: 1, Lyme-Lyme N: 1 and Sejroe-Sejroe N: 2) and titres < 1:800 against antigens included in the routine assay, for a total of 6/288 (2.1%) dogs detected positive only with the expanded MAT assay using a cut-off ≥ 1:800 (**Table 3**). Furthermore, other 35/288 (12.1%) dogs had prevalent MAT titre against at least one of the 18 *Leptospira* antigens not included in the routine MAT assay, with values higher (N: 14) or equal (N: 21) to those obtained against serovars included in the routine MAT assay: Australis-Jalna (N: 7), Cynopteri-Cynopteri (N: 6), Hurstbridge-Hurstbridge (N: 4), Javanica-Javanica (N: 1), Lyme-Lyme (N: 4), Pyrogenes-Pyrogenes (N: 4), Sejroe-Saxkoebing (N: 2), Sejroe-Sejroe (N: 3), Australis-Jalna with Cynopteri-Cynopteri (N: 1), Autumnalis-Autumnalis with Cynopteri-Cynopteri (N: 2) and Cynopteri-Cynopteri with Sejroe-Sejroe (N: 1) (**Online Resource 1**).

Considering the results obtained by the expanded MAT assay with a cut-off ≥ 1:100, the frequency of seropositivity was significantly higher in apparently healthy kennel dogs, followed by owned dogs showing clinical signs potentially associated with *Leptospira* infection or increased CRP serum concentration and lower in apparently healthy blood donor dogs (P = 0.0124, **Table 2**),

199 whereas no significant association was found between seropositivity and clinical status. Frequency
200 of seropositivity was also significantly higher in mixed breed dogs (35/60, 58.3%) compared to
201 purebred ones and in regularly vaccinated dogs compared to not regularly vaccinated ones (P =
202 0.0198 and 0.0009 respectively, **Table 2**). No other significant association was found between the
203 seropositivity to *Leptospira* and the variables analysed (**Table 2**). Differently, no significant
204 association was found between the seropositivity with a cut-off ≥ 1:800 and all the variables
205 analysed, including the clinical and vaccination status (**Online Resource 2**).
206
207 **Discussion**
208 The MAT assay with the expanded 27 antigens panel detected 13/288 (4.5%) more seropositive
209 dogs than the routine MAT assay with the nine antigens panel. Furthermore, 35/288 (12.1%) dogs
210 had prevalent MAT titre against at least one of the 18 *Leptospira* antigens not included in the
211 routine MAT assay.
212 In the routine nine antigens panel, the Australis-Bratislava, Canicola-Canicola, Grippotyphosa-
213 Grippotyphosa, Icterohaemorrhagiae-Copenhageni, Icterohaemorrhagiae-Icterohaemorrhagiae and
214 Pomona-Pomona were the most frequently detected serovars, confirming that these are the most
215 effective antigens for the diagnosis of *Leptospira* infection in dogs in Northern Italy. Indeed, these
216 variants are the most widespread in Europe (Ellis, 2010), and are included in the vaccines currently
217 adopted (Ellis, 2010; Klaasen et al., 2014; Schuller et al., 2015). The remaining three serovars
218 included in the routine nine antigens panel were detected with low frequency and low titres
219 (Ballum-Ballum and Sejroe-Hardjo), or undetected (Tarassovi-Tarassovi), in this study,
220 highlighting its limited importance for the diagnosis of *Leptospira* infection in dogs. However, their
221 inclusion in the antigen panel is justified by the use of the same MAT assay for the diagnosis of
222 leptospirosis in other animal hosts (Tagliabue et al., 2016).
223 Among the serovars not included in the routine nine antigens panel, Australis-Jalna and Pomona-
224 Mozdok serovars were positive only when Australis-Bratislava and Pomona-Pomona, respectively,

were positive and normally with a lower titre, suggesting a probable cross-reaction. Differently, the Sejroe-Saxkoebing and Sejroe-Sejroe serovars were positive more frequently and with higher titre than Sejroe-Hardjo serovar, suggesting that they are more effective for the diagnosis of *Leptospira* infection in dogs and its inclusion in the antigen panel of the MAT assay could be useful. A recent study conducted in Italy reported the usefulness of using the Saxkoebing and Sejroe serovars, in addition to Hardjo, for the MAT assay, identifying a higher number of seropositive dogs in a leptospirosis outbreak in a kennel (Balboni et al., 2022). The Autumnalis-Autumnalis, Cynopteri-Cynopteri, Hurstbridge-Hurstbridge, Lyme-Lyme and Pyrogenes-Pyrogenes serovars were not included in the routine antigen panel and showed a high frequency of positivity. Among these, the Cynopteri serovar is the most interesting as it often showed the highest titre, alone or in association with other serovars. The other four serovars had antibody titres usually less than 1:800 and, when associated with positivity to other serovars, they were rarely those with the highest titre, therefore, although their role in dogs cannot be ruled out with certainty, it is plausible to speculate that they could be the results of non-specific or cross reactions. Serovar Cynopteri and its serogroup are poorly documented in the literature and available data are usually limited to serological reactivity. A seroprevalence of 59% in dogs in Buenos Aires (Argentina) was reported by Tealdo and colleagues (Tealdo et al., 2007). Cynopteri serovar was also reported in dogs in Peru (Siuce et al., 2015) and in Portugal, where it was found to be among the most common reactivity in pigs, sheep and horses (Rocha, 1998). Bats are the maintenance hosts of the Cynopteri serovar (Bharti et al., 2003). While its pathogenicity is not clearly documented in the dog, its infection is clinically relevant in humans (Bharti et al., 2003), with a case reported in Poland (Zwierz et al., 1964). The remaining eight serovars not included in the routine MAT assay evaluated in this study (Bataviae-Bataviae, Celledoni-Celledoni, Hebdomadis-Hebdomadis, Javanica-Javanica, Mini-Mini, Panama-Panama, Ranarum-Ranarum, and Shermani-Shermani) were not detected or sporadically detected with low titres not exceeding 1:800 in association with other serovars, resulting negligible for the diagnosis of *Leptospira* infection in dogs in Northern Italy.

This study confirms that kennels may represent high-risk environments for the diffusion of leptospiral infection in dogs (Balboni et al., 2022), as demonstrated by the significantly higher frequency of seropositivity ≥ 1:100 for kennel dogs than for owned dogs, also confirmed by a significantly higher frequency of seropositivity in mixed breed dogs, mainly coming from kennels. Differently, the significantly higher frequency of seropositivity ≥ 1:100 in regularly vaccinated dogs than in not regularly vaccinated ones is a probable consequence of the positive reaction to the MAT test due to antibodies of vaccine origin.

The main limitation of this study was the lack of a second paired MAT test on the enrolled dogs. This aspect, associated with the typical paradoxical reactions and cross-reactivity that characterise the MAT assay (Levett, 2003; Murray et al., 2011; Smythe et al., 2009; Sykes et al., 2022), could determine a misinterpretation of the highest titres obtained. Indeed, especially if an animal is tested only once, the antigen with the highest titre cannot be considered with certainty the infecting serovar. The lack of a second paired MAT test would have been an important limitation if the main aim of the study was to assess the diffusion of the different serovars in Northern Italy. Otherwise, as the aim of this study was to identify the most effective serovars to include in the MAT antigen panel for the diagnosis of *Leptospira* infection in dogs and because the antibody titre was only interpreted as a measure of seroreactivity, this can be considered a negligible limitation. Nevertheless, seroepidemiological studies involving the analysis of paired serum samples collected at appropriate times (acute and convalescent) in dogs with clinical signs potentially associated with *Leptospira* infection should be performed to more accurately detect which serogroups circulate and cause disease in the canine population.

Conclusions

In general, the antigen panel currently adopted for the routine MAT assay in Northern Italy resulted adequate for the diagnosis of *Leptospira* infection in dogs, both for the serogroups detected and for the choice of the serovar within the serogroup. The main exception concerns the Sejroe serogroup,

with the Saxkoebing and Sejroe serovars that were more effective than Hardjo for diagnosis in dogs and whose inclusion in the antigen panel is recommended. Among other antigens evaluated in this study, Cynopteri serovar was detected with high frequency but, as it was usually in association with other serogroups, its pathogenic role in dogs and public health threats deserve further investigation.

Statements and Declarations

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Authors' contributions: All authors contributed to the study conception and design. Material preparation, data collection and laboratory analysis were performed by Mario D'Incau, Silvia Marchione and Alda Natale. The analysis of the results was performed by Andrea Balboni, Mario D'Incau and Silvia Zamagni. The first draft of the manuscript was written by Andrea Balboni and Silvia Zamagni. Mario D'Incau, Mara Battilani, Laura Lucchese, Elisa Mazzotta and Alda Natale supervised and commented on previous versions of the manuscript. Funding acquisition was made by Alda Natale. All authors read and approved the final manuscript.

Availability of data and material: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval: Ethical approval were waived for this study, because only blood samples taken by clinicians from owned dogs for diagnostic purposes and surplus material derived from blood samples taken by clinicians from kennel dogs undergoing neutering surgery sampled to perform pre-operative profile tests or from donor dogs sampled to perform pre-donation screening tests were used. For all dogs, blood sampling was performed following owner or legal manager of the kennel consent.

Consent to participate: Not applicable.

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Table 1 Panel of eleven *Leptospira* spp. used as live antigens for MAT assay and results obtained.

Serogroup	Serovar	Strain	Routine MAT [a]	Expanded MAT [b]	Dogs tested positive with cut-off ≥ 1:100	Dogs tested positive with cut-off ≥ 1:800	Highest MAT titre
Australis	Bratislava	Riccio 2	■	■	54 (18.7%)	26 (9%)	1:6400
Australis	Jalna	Jalna		■	42 (14.6%)	19 (6.6%)	1:6400
Autumnalis	Autumnalis	Akiyami A		■	25 (8.7%)	9 (3.1%)	1:6400
Ballum	Ballum	Mus 127	■	■	9 (3.1%)	1 (0.3%)	1:800
Bataviae	Bataviae	Swart		■	1 (0.3%)	0 (0%)	1:100
Canicola	Canicola	Alarik	■	■	53 (18.4%)	5 (1.7%)	1:1600
Celledoni	Celledoni	Celledoni		■	0 (0%)	0 (0%)	0
Cynopteri	Cynopteri	3522 C		■	27 (9.4%)	13 (4.5%)	1:6400
Grippotyphosa	Grippotyphosa	Moskva V	■	■	25 (8.7%)	14 (4.9%)	1:6400
Hebdomadis	Hebdomadis	Hebdomadis		■	2 (0.7%)	0 (0%)	1:400
Hurstbridge	Hurstbridge	BUT 6		■	14 (4.9%)	2 (0.7%)	1:1600
Icterohaemorrhagiae	Copenhageni	Wijnberg	■	■	87 (30.2%)	21 (7.3%)	1:6400
Icterohaemorrhagiae	Icterohaemorrhagiae	Bianchi	■	■	59 (20.5%)	14 (4.9%)	1:6400
Javanica	Javanica	Veldrat Bataviae 46		■	7 (2.4%)	3 (1%)	1:800
Lyme	Lyme	10		■	20 (6.9%)	3 (1%)	1:3200
Mini	Mini	Sari		■	0 (0%)	0 (0%)	0
Panama	Panama	CZ 214 K		■	5 (1.7%)	1 (0.3%)	1:800
Pomona	Mozdok	5621		■	15 (5.2%)	5 (1.7%)	1:3200
Pomona	Pomona	Pomona	■	■	15 (5.2%)	8 (2.8%)	1:6400
Pyrogenes	Pyrogenes	Salinem		■	21 (7.3%)	3 (1%)	1:1600
Ranarum	Ranarum	ICF		■	2 (0.7%)	1 (0.3%)	1:800
Sejroe	Hardjo	Hadjoprajitno/g.t. hardjoprajitno	■	■	6 (2.1%)	1 (0.3%)	1:1600
Sejroe	Hardjo	Sponselee/g.t. hardjobovis		■	5 (1.7%)	1 (0.3%)	1:3200
Sejroe	Saxkoebing	Mus24		■	9 (3.1%)	3 (1%)	1:6400
Sejroe	Sejroe	M84		■	16 (5.6%)	3 (1%)	1:3200
Shermani	Shermani	LT 821		■	0 (0%)	0 (0%)	0
Tarassovi	Tarassovi	Mitis-Johnson	■	■	0 (0%)	0 (0%)	0

[a] MAT assay with antigen panel composed by eight serogroups and nine serovars.

[b] MAT assay with antigen panel composed by 20 serogroups and 27 serovars.

Table 2 Descriptive statistics of the dogs included in the study population and comparison between dogs tested positive and dog tested negative by MAT assay with expanded 27 antigens panel adopting a cut-off ≥ 1:100.

Variables	Total	Positive	Negative	P value
Number of dogs	288	141 (49%)	147 (51%)	
Origin				
Owned dogs showing clinical signs potentially associated with *Leptospira* infection or increased CRP serum concentration	217	110 (50.7%)	107 (49.3%)	**0.0124**
Apparently healthy kennel dogs	20	14 (70%)	6 (30%)	
Apparently healthy blood donor dogs	51	17 (33.3%)	34 (66.7%)	
Sex				
Male	108	50 (46.3%)	58 (53.7%)	0.9342
Female	94	45 (47.9%)	49 (52.1%)	
NA	86			
Age [a]	5y [1m-16a]	5y6m [1m-16y]	4y1m [1m-14y4m]	
<1	25	12 (48%)	13 (52%)	0.5451
1-5	66	33 (50%)	33 (50%)	
6-10	49	29 (59.2%)	20 (40.8%)	
>10	15	6 (40%)	9 (60%)	
NA	155			
Breed				
Purebred	133	52 (39.1%)	81 (60.9%)	**0.0198**
Mixed breed	60	35 (58.3%)	25 (41.7%)	
NA	95			
Vaccination				
Yes	94	59 (62.8%)	35 (37.2%)	**0.0009**
No [b]	59	20 (33.9%)	39 (66.1%)	
NA	135			
Clinical status				
Sick	217	110 (50.7%)	107 (49.3%)	0.3725
Apparently healthy	71	31 (43.7%)	40 (56.3%)	

The Chi-squared test were carried out on the positive and negative dogs. Not available data was excluded to statistical analysis. Data are reported as n (%).

[a] Data are reported as median [range] and in four age classes, statistical analysis was carried out on categorical age classes.

[b] Dog not vaccinated or vaccinated for more than 12 months (Day et al., 2016).

Values in bold indicate statistical significance. m: months. NA: not available. y: years.

Table 3 Comparison between routine MAT results and expanded MAT results.

Cut-off ≥ 1:100					Cut-off ≥ 1:800				
		Expanded MAT [b]					Expanded MAT [b]		
		positive	negative	total			positive	negative	total
Routine	**positive**	128 (44.5%)	0 (0%)	128 (44.5%)	**Routine**	**positive**	46 (16%)	0 (0%)	46 (16%)
MAT [a]	**negative**	13 (4.5%)	147 (51%)	160 (55.5%)	**MAT [a]**	**negative**	6 (2.1%)	236 (81.9%)	242 (84%)
	total	141 (49%)	147 (51%)	288 (100%)		**total**	52 (18.1%)	236 (81.9%)	288 (100%)

[a] MAT assay with antigen panel composed by eight serogroups and nine serovars.

[b] MAT assay with antigen panel composed by 20 serogroups and 27 serovars.