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Original Article

Prospective evaluation of rapid point-of-care tests for the diagnosis of acute leptospirosis in dogs

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ABSTRACT

The early diagnosis of acute leptospirosis is still a major challenge in dogs. The aim of this prospective study was to evaluate the suitability of two in-clinic tests detecting anti-leptospiral IgM and IgG antibodies in diagnosing canine leptospirosis. The performances of the two rapid tests were compared to the microscopic agglutination test (MAT) carried out on acute sera and to diagnostic criteria adopted in this study to confirm leptospirosis infection (MAT upon admission, convalescent MAT and quantitative real-time PCR on blood and/or urine). The dogs were enrolled on the basis of reported exposure to known risk factors and clinical presentation (acute kidney injury and/or systemic inflammatory response syndrome with multi-organ damage). Eighty-nine dogs included in the study were sub-grouped on the basis of the results of the diagnostic criteria adopted: (1) confirmed leptospirosis cases (42/89 dogs); (2) negative leptospirosis cases (36/89 dogs); and (3) unconfirmed leptospirosis cases (11/89 dogs). The results supported the usefulness of the two rapid diagnostic tests as a first in-clinic screening tool for suspected leptospirosis; positive results in the in-clinic tests in dogs with suggestive clinical and laboratory signs strongly indicated acute leptospirosis, while negative results required additional diagnostic investigation to exclude the infection. Confirmatory tests recommended for canine leptospirosis are still necessary in addition to the use of rapid in-clinic tests.

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Introduction

Canine leptospirosis is a zoonotic disease of global importance with variable clinical manifestations (Mastrorilli et al., 2007; Major et al., 2014). The early diagnosis of acute leptospirosis is still a major challenge in both human beings and dogs, mainly due to the reported limitations of the diagnostic tests available (Smits et al., 2001; Schuller et al., 2015; Doungchawee et al., 2017).

The microscopic agglutination test (MAT) detects anti-leptospiral immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies, and represents the reference method for the serological diagnosis of leptospirosis. Anti-leptospiral antibodies are detectable 7–10 days after the onset of the disease in both human beings and dogs (Fraune et al., 2013; Schuller et al., 2015; Doungchawee et al., 2017; Gloor et al., 2017). Since MAT results can be non-diagnostic in the early phase of disease (within 7 days after infection), evaluation of a convalescent sample to detect seroconversion is recommended (Levett, 2001; Schuller et al., 2015; Doungchawee et al., 2017; Lizer et al., 2018). Paired serological testing may be impossible to carry out in some cases, owing to sudden death or failure to follow up, potentially leading to a false negative diagnosis in the acute setting (Fraune et al., 2013; Schuller et al., 2015; Doungchawee et al., 2017).

Molecular diagnosis of leptospirosis can be achieved using blood and urine samples by means of conventional PCR or real-time quantitative PCR (qPCR); however, pre-analytical conditions, previous treatment with antimicrobial agents and the time course of the infection can reduce the sensitivity of this assay (Fraune et al., 2013; Schuller et al., 2015). ELISAs for detection of anti-leptospiral IgM have been developed for early diagnosis of leptospirosis (Dahal et al., 2016; Penna et al., 2017).

Rapid point-of-care tests detecting IgM and/or IgG have been developed as screening tools for canine leptospirosis (Lilenbaum et al., 2002; Abdoel et al., 2011; Subathra et al., 2011; Winzelberg et al., 2015; Kodjo et al., 2016; Gloor et al., 2017; Lizer et al., 2017). These tests have not replaced testing paired sera by MAT for confirming the diagnosis but, being rapid, convenient and user friendly, they can offer the advantages of providing a diagnostic

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indication before the availability of MAT and the qPCR results (Panwala et al., 2015). In addition, point-of-care tests able to quantify IgM can be of particular value for the earlier detection of leptospiral antibodies in dogs when the MAT might be negative (Doungchawee et al., 2017). The aim of the present study was to evaluate the diagnostic accuracy of two rapid tests for the in-clinic evaluation of acute canine leptospirosis compared with the MAT carried out on acute sera and the diagnostic criteria adopted in this study to confirm leptospiral infection.

Materials and methods

Design, inclusion criteria and study groups

This was a prospective study conducted at the Veterinary Teaching Hospital of the University of Bologna (January 2015–January 2017) and approved by the Scientific and Ethical Committee of the University of Bologna (approval number 751). Dogs with suspected acute leptospirosis infection were included on the basis of: (1) reported exposure to known risk factors (Schuller et al., 2015; Grayzel and DeBess, 2016); (2) acute kidney injury (AKI) (Cowgill and Langston, 2011) and/or systemic inflammatory response syndrome (SIRS) (Hauptman et al., 1997); and (3) evidence of damage/dysfunction to at least one organ other than the kidney (Kenney et al., 2010; Tangeman and Littman, 2013). Dogs sharing the same household with dogs testing positive for leptospiral infection were also included in the study. Dogs vaccinated for leptospirosis in the 15 weeks preceding hospitalisation were excluded if the MAT revealed antibodies against a leptospiral serogroup included in the vaccine administered (Martin et al., 2014; Lizer et al., 2017).

Leptospiral infection was confirmed by a combination of tests representing the diagnostic criteria adopted in this study (Schuller et al., 2015): (1) MAT at the time of hospital admission; (2) convalescent MAT; and/or (3) qPCR on blood and/or urine samples. On the basis of diagnostic test results, dogs were grouped as: (1) confirmed leptospirosis cases (CLCs), i.e. dogs with a positive MAT $\geq$ 1:800 on a single serum sample and/or a four-fold increase in titre in paired sera (Kohn et al., 2010; Fraune et al., 2013), and/or positive qPCR on blood and/or urine samples (Schuller et al., 2015); (2) negative leptospirosis cases (NLCs), i.e. dogs with a negative MAT (<1:800) in paired sera and negative qPCR results; and (3) unconfirmed leptospirosis cases (UCLCs): dogs with an incomplete diagnostic protocol (biological specimens unavailable due to incomplete sampling, inadequate storage or early death). All dogs were tested using rapid in-clinic immunodiagnostic assays, but UCLCs were excluded from the statistical comparison of assays.

Microscopic agglutination test

The MAT was carried out at the National Reference Laboratory for Leptospira (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, IZSLER), Bologna, Italy, and was able to determine titres against eight leptospiral serogroups (Australis, Canicola, Ballum, Grippotyphosa, Icterohaemorrhagiae, Pomona, Sejroe and Tarassovi).

Molecular detection of Leptospira *spp.*

DNA was extracted from blood and urine using the NucleoSpin Tissue Mini Kit (Macherey-Nagel). In some cases, specimens were stored at −20 °C before extraction. Urine samples were centrifuged and the DNA was extracted from the supernatant without pH neutralisation ('non-neutralised urine samples'; see Appendix: Supplementary material). When urine samples were received at the laboratory within 90 min of collection, DNA was also extracted from another aliquot of urine supernatant after pH neutralisation (>7) by adding sterile phosphate buffered saline (PBS) ('neutralised urine samples'). A modified SYBR green qPCR targeting the gene encoding the major outer membrane protein *LipL32* (Stoddard et al., 2009) was used to detect pathogenic *Leptospira* spp. (see Appendix: Supplementary material).

Rapid immunodiagnostic tests

Two rapid tests for in-clinic diagnosis of canine leptospirosis were performed using acute canine serum samples: (1) rapid diagnostic test A (RDT-A): Witness Lepto (Zoetis), which uses whole cell antigen extracts of *Leptospira kirschneri* serovar Gryppotyphosa and *L. interrogans* serovar Bratislava to detect canine IgM, has a sensitivity of 98% and a specificity of 93.5% (Kodjo et al., 2016; Lizer et al., 2017); no clear manufacturer's accuracy data were available; and (2) rapid diagnostic test B (RDT-B): SNAP Lepto (IDEXX), which detects antibodies against Lip32 antibodies by ELISA; according to the manufacturer, this test has a sensitivity of 82% and a specificity of 96% (Winzelberg et al., 2015).

Statistical analysis

At least 26 dogs for each group was considered to be adequate in order to achieve 90% statistical power, based on the assumption of a 1:1 confirmed to negative case ratio and a 5% type I error rate (α). After checking for normality (Shapiro–Wilk test), the data were evaluated using standard descriptive statistics. The diagnostic performances of the two rapid immunodiagnostic tests were evaluated in the CLC and the NLC groups; UCLC cases were excluded from all analyses. Using receiver operating characteristic (ROC) curves, results were compared to the MAT performed on acute sera and the diagnostic criteria adopted for this study. Significance was set at $P < 0.05$. An inter-rater agreement statistic (Cohen's kappa coefficient) was calculated to compare the results obtained by the diagnostic criteria adopted for this study and the results obtained by the MAT carried out on acute sera and the two rapid immunodiagnostic tests. The statistical analysis was performed using MedCalc Statistical Software version 16.8.4. On the assumption that all diagnostic tests are imperfect, the MAT on acute sera, RDT-A, RDT-B and qPCR were also compared using Bayesian latent class modelling to estimate prevalence, sensitivity, specificity, and positive and negative predictive values (Eugene et al., 2015; Niloofa et al., 2015).[1]

Results

Eighty-nine dogs were included in the study (Table 1) and 87 dogs were tested for the presence of *Leptospira* spp. DNA by qPCR (Table 2).

Confirmed leptospirosis cases

Forty-two (47.2%) of 89 dogs were classified as CLCs. Eighteen of 42 (42.9%) dogs had an inadequate vaccination history (Day et al., 2016), while the remaining 24 dogs had been adequately vaccinated with a bivalent (23/42, 54.7%) or a tetravalent (1/42, 2.4%) leptospirosis vaccine 15–32 weeks before hospitalisation. Most (88.1%) dogs classified as CLCs had access to an outdoor environment; 21/42 (50.0%) dogs lived in a rural environment (countryside or farm), 12/42 (28.6%) dogs lived indoors but had occasional access to a rural environment, 4/42 (9.5%) were hunting dogs, 4/42 (9.5%) were dogs living in flats with access only to an urban environment and 1/42 (2.4%) was a kennel dog. Most were hospitalised for AKI (34/42, 81.0%) or SIRS associated with different dysfunctional organs (4/42, 9.5%); the exceptions were 4/42 (9.5%) cohabiting dogs, which were asymptomatic and were managed as 'out-patients'.

The diagnosis of acute leptospirosis was established in 32/42 (76.2%) dogs with a positive MAT (titre $\geq$ 800) at the time of hospital admission and 4/42 (9.5%) dogs with a four-fold increase in the MAT titre. At the time of admission, 24/32 (75.0%) seropositive dogs showed partial cross-reactivity among the serogroups tested; the highest MAT titres were more frequently attributable to the Australis serogroup, followed by the Grippotyphosae and Icterohaemorragie serogroups (Fig. 1).

A convalescent MAT was carried out in 28/42 (66.6%) dogs 14–21 days after admission to the hospital; early death precluded analysis of paired sera in the remaining 14/42 (33.3%) dogs. Convalescent MAT titres were similar to acute titres in 24/28 (85.7%) dogs. Seroconversion was documented in 4/28 (14.3%) dogs; in the convalescent MAT, 2/4 dogs had higher titres against the Australis serogroup and 2/4 had higher titres against the Grippotyphosa serogroup.

Leptospiral DNA was detected by qPCR in blood and/or urine samples of 9/42 (21.4%) dogs in the CLC group (see Appendix: Supplementary material). Five of 42 (11.9%) dogs had positive blood samples and 5/42 (11.9%) dogs had positive urine samples; one dog had both positive blood and urine samples. Of the five dogs with positive urine samples, three were positive in the non-neutralised urine sample only, one was positive in the neutralised urine sample only and one was positive in both non-neutralised and neutralised urine samples (see Appendix: Supplementary

[1] See: Modelling of Infectious Disease Centre. http://mice.tropmedres.ac (accessed 13 May 2018).

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