Evaluation of a rapid IgM detection test for diagnosis of acute leptospirosis in dogs

J. Lizer, M. Grahlmann, H. Hapke, S. Velineni, D. Lin, B. Kohn

Recently, a lateral flow assay (LFA) for detection of Leptospira-specific IgM in canine sera became commercially available in Europe. The present study aims to evaluate the diagnostic performance of this assay using canine sera from a collection of diagnostic accessions. Diagnostic sensitivity was assessed by testing 37 acute-phase and 9 corresponding convalescent-phase sera from dogs with a confirmed diagnosis of leptospirosis. Specificity was determined by testing sera from sick dogs with non-leptospiral infections (n=15) and healthy dogs with an incomplete history of vaccination (n=45). During acute phase of illness, LFA scored positive for 28/37 sera with a sensitivity of 75.7 per cent while only 9/37 (24.3 per cent) samples were positive on microscopic agglutination test. The specificity of the LFA was 98.3 per cent (59/60). This test showed 89.7 and 100 per cent overall agreements with clinical diagnosis for acute-phase and convalescent-phase sera, respectively. The impact of vaccination on the LFA was also determined and vaccine-stimulated IgM responses were negative in 19/25 (76 per cent) dogs at 12 weeks post vaccination. In conclusion, the LFA is a rapid and reliable test for early detection of Leptospira-specific IgM during acute phase of canine leptospirosis. However, interpretation of a positive result must be made in the context of clinical signs and vaccination history.

Leptospirosis is a spirochetal zoonosis of global concern, caused by pathogenic serovars belonging to the genus Leptospira. The disease is contagious and known to affect human beings, domestic animals and wildlife (Levett 2001, Vinetz 2001, Bharti and others 2005). Seroprevalence studies have indicated exposure of dogs to serovars belonging to Leptospira interrogans and Leptospira kirschneri are widespread in North America (Sykes and others 2010) and Europe (Schuller and others 2015). The most frequently circulating serovars in affected dogs have been Canicola, Icterohaemorrhagiae, Grippotyphosa, Pomona, Bratislava and Autumnalis (Moore and others 2006, Ellis 2010). The syndrome associated with canine leptospirosis is characterised not only by a wide range of acute clinical features including anorexia, lethargy, abdominal pain, vomiting, pyrexia, myalgia, renal failure, pulmonary haemorrhage and icterus but also chronic carriage that might result in reproductive losses and uveitis (Greene and others 2006, Van de Maele and others 2008, Gallagher 2011). Leptospires often colonise the proximal convoluted renal tubules and shed in the urine of infected animals for extended periods and infection may spread via urine-contaminated environment. Hence, early diagnosis of leptospirosis is crucial for prompt initiation of antibiotic therapy, which may prevent progression of disease as well as zoonotic transmission.

The laboratory diagnosis of leptospirosis is generally accomplished by isolation of leptospires from biological specimens, seroconversion using paired sera (Faine 1995, Sykes and others 2010) or a single titre of ≥1:800 (Miller and others 2008) in microscopic agglutination test (MAT) and PCR using anti-coagulated blood and urine (Harkin and others 2003, Xu and others 2014). Isolation and cultivation of leptospires from clinical samples is difficult. Although PCR is a useful tool for diagnosis during early stages of infection, it requires special expertise, is unable to distinguish between live and dead organisms and is highly susceptible to laboratory contaminations. MAT continues to remain as the accepted serological ‘gold standard’ method; however, existing serological tests including MAT and ELISA (Adler and others 1980) are time-consuming and require maintenance of a battery of pathogenic serovars, special equipment and trained personnel to perform the test (Faine and others 1999, Levett 2001). Thus there is a need for a rapid, accurate and easy-to-perform point-of-care test for the diagnosis of acute leptospirosis in a clinical setting. IgM antibodies to Leptospira are ideally suited for the detection of acute leptospirosis. They appear early in the course of infection, about four to six days after the onset of clinical disease, but they are only transitionally produced after vaccination (Adler and Faine 1978, Hartman 1984). The latter is an important consideration given the widespread use of multivalent leptospiral vaccines in North America and Europe in the canine population. Over the years, lateral flow assays (LFAs) for the rapid detection of Leptospira-specific IgM antibodies in human (Smits and others 2001, Sehgal and others 2005, Vanithamani and others 2015) and canine sera (Abdoel and others 2011) have
been developed. Recently, a point-of-care IgM-based LFA (WITNESS Lepto, Zoetis) for rapid diagnosis of acute leptospirosis in dogs was described (Kodjo and others 2016).

The objectives of the present study included (a) evaluation of the diagnostic performance of this commercially available LFA using a set of well-characterised archived sera from client-owned dogs and (b) assessment of the impact of vaccination on the test performance.

Materials and methods

Leptospira strains

Leptospira reference serovars (n=17) used as live antigens in MAT included Canicola, Pomona, Grippotyphosa, Australis, Bratislava, Ballum, Copenhageni, Autumnalis, Tarassovi, Pyrogenes, Javanica, Sejroe, Icterohaemorrhagiae, Hebdomadis, Hardjo, Bataviae, Saxkoebing and were maintained by regular subculturing in Ellingham-McCullough-Johnson-Harris bovine serum albumin-Tween 80 medium (Federal Institute for Risk Assessment, Berlin).

Microscopic agglutination test

MAT was performed as described elsewhere (Kohn and others 2010) at the Federal Institute for Risk Assessment, Berlin. Briefly, in a 96-well round bottom polystyrene microwell plate, 25 μl of sera diluted twofold starting at 1:25 was incubated with equal volumes of each live reference serovar separately. The serovar control included 25 μl of live organism without addition of antibody. The endpoint titre was the highest dilution of the serum in which 50 per cent of the leptospiral cells were agglutinated compared with the control.

A positive cut-off titre of ≥1:800 was considered for all non-vaccine sera. In the case of sera from vaccinated dogs, positive cut-off titres of ≥1:800 and ≥1:3200 were considered for non-vaccine and vaccine serovars, respectively. Dependent on the vaccine used, serovars Canicola, Icterohaemorrhagiae and Copenhageni, which cross-reacts with the latter, were considered as vaccine serovars. Additionally, a fourfold or greater rise in titres on paired sera, regardless of vaccination status, was considered diagnostic (Kohn and others 2010).

PCR

Before January 2013, a multiplex PCR assay using combined sets of primers G1, G2 and B64-I, B64-II as described previously (Gravekamp and others 1993) was used to detect leptospiral DNA in urine and EDTA anticoagulated blood of dogs involved in the study. Subsequently, the PCR assay targeting lipL32 using 270F and 692R primers was performed on urine and canine blood samples (Levett and others 2005). Clinical specimens were centrifuged at 20,000 rpm for 10 minutes and DNA was then isolated from pellets using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. Reaction mix containing water and DNA from reference strains L. interrogans serovar Icterohaemorrhagiae strain RGA and L. kirschneri serovar Grippotyphosa strain Moskva V served as negative and positive controls, respectively. Amplified products were resolved on 1.5–2.0 per cent agarose gel and visualised after staining with ethidium bromide.

Sera

Sera used in the study were from a collection of diagnostic accessions between July 2012 and April 2014 submitted to the Freie Universität Berlin, Germany. A combination of clinical signs, MAT and/or a positive blood or urine PCR were used as standard criteria for clinical diagnosis of leptospirosis upon receipt of samples. Based on standard criteria, sera (n=107) from 97 client-owned dogs were categorised into group (1) dogs with a confirmed diagnosis of leptospirosis (n=57); group (2) sick dogs diagnosed with non-leptospiiral infections (n=15) and group (3) healthy dogs with incomplete history of vaccination (n=45) (Table 1). Sick dogs from group 2 were diagnosed with Cushing’s disease (n=5), immune-mediated haemolytic anaemia (n=5), hepatopathy (n=2), pancreatitis (n=2), ehrlichiosis (n=1), lymphatic leukaemia (n=1), malignant lymphoma (n=1), immune-mediated thrombocytopenia (n=1) and epilepsy (n=1). Convalescent-phase sera from group 1 (n=9) and group 2 (n=1) were received 12–19 days after the first serum sample. Dogs in groups—1 to 3 comprised of 53 breeds or mixed breeds with ages ranging from 4 months to 17 years.

A group of 25 dogs (group 4) were vaccinated (Nobivac Lepto, Merck Animal Health) twice subcutaneously at an interval of four weeks. Sera were collected at the time of initial vaccination and 4, 12 and 26 weeks post vaccination (WPV).

Lateral-flow assay

The LFA (WITNESS Lepto, Zoetis) was performed retrospectively in 2015 according to the manufacturer’s instructions by a technician who was blinded to the outcome of clinical diagnosis. Briefly, the test consisted of a nitrocellulose strip flanked at one end by a sample pad and other end by an absorption pad, housed in a plastic cassette. The conjugate pad located adjacent to the sample pad holds dried conjugate consisted of colloidal gold-labelled anti-dog IgM antibody. The strip contained a test line consisting of whole cell extracts of serovars Grippotyphosa and Bratislava and a control line consisting of an unrelated antigen that binds a second gold conjugate not specific to Leptospira. Five microliters of serum were added to the sample well using a capillary pipette provided in the kit, followed by four drops of chase buffer. The results were read after 10 minutes at ambient temperature. Leptospira-specific IgM antibodies in sera first bind to the colloidal gold-labelled antidog IgM to form complexes. The gold-antibody complexes accumulate on the test line, resulting in the formation of a red line, which indicates a positive result, whereas a negative result was interpreted by the absence of a test line. The formation of a control line on all tests indicated proper function of the test.

Statistical analyses

Sensitivity and specificity with 95 per cent Jeffrey’s CIs were calculated by comparing the LFA to clinical diagnosis of leptospirosis as accomplished by standard criteria. Sensitivity was obtained from the proportion of true positive samples from dogs with a confirmed diagnosis of leptospirosis (group 1), and specificity was calculated from the proportion of true negative samples from sick dogs with an infection other than leptospirosis and healthy controls (groups 2 and 3). Results

Diagnostic performance of the LFA was determined by testing well-characterised canine sera from a collection of diagnostic accessions (Table 1). A set of 37 acute-phase sera and 9 corresponding convalescent-phase sera were available from 57 dogs that were diagnosed with leptospirosis (group 1). The LFA scored positive for 28/37 acute-phase sera with a sensitivity of 75.7 per cent (95 per cent CI 60.3 to 87.2 per cent) (Table 2), while only 9/57 (24.3 per cent) of these sera were positive on MAT (Table 3). These sera with a low or negative MAT result had a minimum of fourfold rise in titre for at least one serovar on convalescent MAT or positive PCR results (data not shown).

| TABLE 1: Categories of sera based on standard criteria for clinical diagnosis of leptospirosis |
|------------------|------------------|------------------|
| Group | Cases | Acute phase | Convalescent phase |
| 1 | Dogs with a confirmed diagnosis of leptospirosis | 37 | 9 |
| 2 | Sick dogs diagnosed with non-leptospiiral infection | 15 | 1 |
| 3 | Healthy dogs with incomplete history of vaccination | 45 | – |

2 of 5 | Veterinary Record | 10.1136/vr.104134
Among nine false-negative sera by the LFA, only one serum sample had a high MAT titre (Bratislava 1:800; Table 3). All nine convalescent-phase sera from group 1 tested positive (100 per cent) on the LFA, whereas only five of the corresponding acute-phase sera were LFA positive (55.6 per cent).

The LFA results were negative for all 15 sera from sick dogs diagnosed with non-leptospiral infection (group 2) and for 44/45 healthy dogs with incomplete history of vaccination with a single false-positive sample (group 3), resulting in a test specificity of 98.3 per cent (95 per cent CI 92.5 to 99.8 per cent) (Table 2). The only convalescent-phase serum available for a dog from group 2 was also negative on the LFA. The overall test agreements with the final clinical diagnosis for acute-phase and convalescent-phase sera were 89.7 per cent (87/97) and 100 per cent (9/9), respectively (Table 2).

All 25 prevaccine sera (day 0) from group 4 were negative on the LFA, whereas 16/25 (64 per cent) sera were positive at 4 WPV and decreased to 6/25 (24 per cent) at 12 WPV. Five vaccine sera positive at 12 WPV were negative on the LFA at 26 WPV, and the sixth serum sample was not available for testing.

**Discussion**

Clinical diagnosis of leptospirosis in dogs is often complex and mistaken for other infectious diseases because of its protein manifestations. Hence, definitive diagnosis must be based on the combination of clinical signs and laboratory-based tests. Difficulties in the isolation of leptospires and lack of availability of PCR or serological tests in veterinary clinics entail reliance on diagnostic laboratories (Levett 2001). The MAT, which is the most widely used serologic test, can be negative in the early course of illness (Faine 1982) and is also associated with further problems that include cross-reactivity between pathogenic serovars and poor standardisation of the assay in laboratories (Chappel and others 2004). This necessitated development of a rapid test that accurately detects the causative serovar or its DNA or specific antibody that would be valuable in guiding treatment in early stages of infection. IgM-based ELISAs have been developed (Adler and others 1980, Vedhagiri and others 2015) with a number of modifications including dipstick (Gussenhoven and others 1997, Sehgal and others 1999) and lateral flow platforms for point-of-care use during the acute phase of illness (Levett and others 2001, Smit and others 2001, Sehgal and others 2003, Abdoel and others 2011, Vanithamani and others 2015). Recently, a point-of-care LFA (WITNESS Lepto, Zoetis) for rapid diagnosis of acute leptospirosis in dogs became commercially available in Europe (Kodjo and others 2016). In the present study, the performance of the LFA was assessed in a different geographical setting than that described earlier (Kodjo and others 2016), using well-characterised archived canine sera. Out of 37 sera that were collected early in the course of infection, LFA detected *Leptospirosis*-specific IgM antibodies in 28 sera (Tables 2 and 3). The authors’ ongoing studies showed that the LFA detected *Leptospirosis*-specific IgM antibodies stimulated as early as four days after experimental infection with *L. kirschneri* serovar Grippotyphosa in naive dogs (Lizer and others, unpublished data). The MAT scored positive only in 9 of 37 serum samples and these results are expected as it is well known that MAT has limited diagnostic value during the first week of illness (Thiermann 1984, Sehgal and others 1999, Vijayachari and others 2001). Furthermore, ELISA (Adler and others 1980), an immunoblot assay (Dongchawee and others 2008) and a dipstick method (Sehgal and others 1999) had higher sensitivity in detecting IgM during the first week of illness compared with MAT. Low sensitivity of MAT in acute phase of disease could possibly be explained by the fact that the agglutination of leptospires by clinical sera is dependent on the magnitude of circulating IgM specific to leptospiral lipopolysaccharide (Cumberland and others 1999, Guerreiro and others 2001), whereas multiple IgM-specific epitopes present in the extracted antigen used in the LFA presumably contributed towards higher sensitivity.

Earlier studies also have revealed that the overall sensitivity of MAT was improved by testing paired sera at an interval of two to three weeks and had more diagnostic significance than relying on the results from a single sample (Bajani and others 2003, Sykes and others 2010, Miller and others 2011, Schuller and others 2015). As expected and similar to MAT, the number of positive sera detected by the LFA also increased when paired sera were tested. The LFA detected *Leptospirosis*-specific IgM antibodies in 9/9 convalescent-phase sera tested from group 1 (100 per cent), while only five of the corresponding acute-phase sera were positive (55.6 per cent). This outcome suggests that the false-negative LFA results were due to lack of an adequate interval for primary immune response after onset of symptoms. If clinical suspicion of leptospirosis still remains despite a doubtful negative result, it is advisable that veterinarians should consider retesting a second serum sample on LFA drawn after an interval of one to two weeks (Sykes and others 2010) and other dogs through results from experimental infection studies suggest that the animal can be retested on the LFA as early as three to seven days after the initial test (Lizer and others, unpublished data). Additionally, it is sensible to test the anticoagulated blood sample by PCR. However, testing a second serum sample by convalescent MAT provides valuable information about the infecting serogroup for epidemiological purposes.

A false-positive LFA result in non-infected dogs may occur as a result of previous vaccination and in rare occasions, past exposure, even though this test primarily detects IgM class antibodies. In response to vaccination, most dogs develop relatively low *Leptospirosis*-specific IgM titres (1:100 to 1:400) that persist for up to 1–3 months (Bolin 1996) and very few dogs develop high titres that may persist even up to 12 months (Klaassen and others 2003). These vaccine-stimulated antibodies may confound interpretation of a positive test result in a dog with clinical signs suggestive of leptospirosis. In the present study, the LFA detected vaccine-stimulated IgM in only 6/25 (24 per cent) dogs at 12 WPV and none of the five dogs tested were positive by 26 WPV. Interpretation of a positive result on the LFA must be considered

**Table 3: Comparative analysis of lateral flow assay (LFA) and microscopic agglutination test (MAT) results by testing acute-phase sera from dogs with a confirmed diagnosis of leptospirosis**

<table>
<thead>
<tr>
<th>Test</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA positive</td>
<td>8</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td>LFA negative</td>
<td>1</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>28</td>
<td>37</td>
</tr>
</tbody>
</table>

*A fourfold or greater rise in titres on paired sera was considered diagnostic. If a single serum sample was available, the positive cut-off titre was set as >1:800 for all non-vaccine sera and in the case of vaccine sera, positive cut-off titres of ≥1:800 and ≥1:3200 were considered for non-vaccine and vaccine serovars, respectively.*

10.1136/vr.104134 | *Veterinary Record* | 3 of 5
within the context of the recent vaccine, especially if the dog was vaccinated within 12 weeks.

In conclusion, data suggest WITNESS Lepto to be a valuable diagnostic tool for rapid and early detection of *Leptospira*-specific IgM antibodies in acute-phase sera of clinically suspected dogs. It requires no specialised equipment and can be performed in veterinary clinics by relatively facile operator expertise. Although additional studies are needed to determine the end-point for assay interferences from immune response due to vaccination against *Leptospira* species in dogs, the LFA has the potential to minimise confounding positive results due to interferences generated from vaccine-stimulated antibody between 12 and 26 WFP. However, a positive test result must be interpreted within the context of clinical signs and history of vaccination before making a definitive diagnosis. Future studies that evaluate sensitivity and specificity of this test with a larger sample size, as well as its diagnostic potential in detecting specific immune responses stimulated by the most common canine leptospirosis serovars without discrimination, would be useful to further characterise the diagnostic utility of this test.

Acknowledgements

Preliminary results were presented as an Abstract at the 26th Congress of the European College of Veterinary Internal Medicine – Companion Animal, Gotteborg, Sweden, 8–10 September 2016.

Contributors

All authors shared equal input in the study design, analysis and interpretation of data, writing of the report, and the decision to publish.

Funding

This work was partially funded by Zoetis.

Competing interests

JL, SV and DL are employed by Zoetis and WITNESS Lepto is a product of the company with a business and/or financial interest.

Ethics approval

Sera from client-owned dogs were diagnostic accessions submitted and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Open Access

This is an Open Access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

References


Detection of LipL32-specific IgM by ELISA in sera of patients with a clinical diagnosis of leptospirosis. *Pathogens and Global Health*. 107, 130–135


Evaluation of a rapid IgM detection test for diagnosis of acute leptospirosis in dogs

J. Lizer, M. Grahlmann, H. Hapke, S. Velineni, D. Lin and B. Kohn

Veterinary Record published online February 24, 2017

Updated information and services can be found at:
http://veterinaryrecord.bmj.com/content/early/2017/02/24/vr.104134

These include:

References
This article cites 38 articles, 12 of which you can access for free at:
http://veterinaryrecord.bmj.com/content/early/2017/02/24/vr.104134

Open Access
This is an Open Access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections
Open access (120)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/