Duration of immunity of a multivalent (DHPPi/L4R) canine vaccine against four Leptospira serovars

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\section*{A B S T R A C T}

Despite effective vaccines against common Leptospira serovars, the development of new products with long duration of immunity is still important to protect dogs against leptospirosis. The results from four challenge studies performed one year after vaccination of dogs with a multivalent vaccine containing four Leptospira antigens are reported. Six week old dogs received two vaccinations, three weeks apart, and were challenged 367 days later. Clinical observations were recorded, while blood (culture, biochemistry and haematology), urine (culture) and liver and kidney (culture) samples were collected throughout the study or at necropsy.

All control dogs remained seronegative until challenge, when they seroconverted. Antibody titres to \textit{Leptospira} antigens were seen in vaccinated dogs 21 days after first vaccination and peaked three to six weeks after the second vaccination. Titres decreased in all studies over the following 12 months, until challenge when anamnestic responses were observed. In all studies control dogs demonstrated various abnormal clinical signs, while no vaccinated dogs were affected; differences between groups were only significant following \textit{L. bratislava} challenge. Analysis of blood cultures showed all control and five of the 24 vaccinated dogs were \textit{Leptospira} positive after challenge; all studies showed significant differences between treatment groups in mean number of days with positive cultures. Significant differences between vaccinated and control groups in mean number of days with positive urine cultures were also observed, with all non-vaccinated and one vaccinated dog \textit{Leptospira} positive. The urine culture positive vaccinated dog also gave positive culture from kidney and liver samples. All except one control dog also showed positive \textit{Leptospira} isolation from kidney or liver, with significant differences between vaccinated and control groups observed.

The results demonstrate that administration of a new vaccine to six week old puppies induces immunity which is still effective up to one year later as demonstrated by challenge.

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\section*{1. Introduction}

Leptospirosis in dogs has a worldwide distribution, infection by \textit{Leptospira} resulting in illness of varying severity with the outcome depending on the infecting strain and the host immune response [1]. The genus \textit{Leptospira} includes free-living and pathogenic forms [2] and has been divided into approximately 20 species based on DNA hybridisation [3], and these 20 species subsequently classified into approximately 280 serovars based on antigenic relatedness [4]. Of these serovars, approximately eight to 10 are thought to be important in dogs [5], with seroprevalence data from North America [6] and Europe [7] indicating that \textit{L. interrogans} serovars \textit{icterohaemorrhagiae} and \textit{canicola} are most commonly associated with leptospirosis.

As \textit{L. interrogans} serovars \textit{canicola} and \textit{icterohaemorrhagiae} have historically shown the highest prevalence they have been included in bivalent vaccines for a number of years. However, there is increasing epidemiological evidence that other serovars are involved in canine leptospirosis [6,7], and those resulting in clinical disease include \textit{L. kirschneri} serovar \textit{grippotyphosa}, and \textit{L. interrogans} serovars \textit{pomona}, \textit{autumnalis} and \textit{bratislava} [1,6,7]. Therefore, there is an emerging need to produce new, multivalent \textit{Leptospira} vaccines; with two new products recently approved in Europe [8,9].

In this paper we describe the duration of immunity of a new canine vaccine containing core viral components and four \textit{Leptospira} (\textit{icterohaemorrhagiae}, \textit{canicola}, \textit{bratislava} and \textit{grippotyphosa}) antigens. Dogs were challenged one year after vaccination with different strains of each \textit{Leptospira} and the impact of vaccination
on clinical parameters, serology and re-isolation of *Leptospira* was compared to control dogs in each study.

2. Materials and methods

Dogs received two vaccine doses three weeks apart and were then challenged approximately one year later with heterologous strains of *L. interrogans* serovars *canicola*, *icterohaemorrhagiae* and *bratislava* or *L. kirscheni* serovar *grippotyphosa*. All four studies were designed to be compliant with the European Pharmacopeia monograph 01/2008:0447 for inactivated canine leptospirosis vaccines, text which defines the minimum animal numbers and sampling period required. Each of the four studies was identical in design, apart from the challenge material administered—the sections below therefore describe procedures in a single representative study.

2.1. Animals

Twelve specific pathogen free (SPF) beagle puppies aged six weeks old were enrolled onto the study and allocated to one of two treatments—six dogs received two doses, three weeks apart, of minimum titre vaccine while the remaining six dogs received sterile water. Puppies were free of specific antibodies against the principal serovars of *Leptospira* as determined by micro-agglutination test [10].

The studies were conducted in accordance with the Act on Animal Health and Animal Welfare of The Czech Republic, and had been reviewed by Bioveta a.s. and Zoetis ethical review committees.

2.2. Vaccine

An experimental vaccine batch was produced which contained live canine distemper virus, canine parainfluenza virus, canine adenovirus type 2, canine parvovirus strain 2b (DHPPI) and inactivated *L. canicola*, *icterohaemorrhagiae*, *bratislava* and *grippotyphosa*, and rabies virus (L4R). The DHPPI fraction was freeze-dried while the L4R fraction was liquid and contained adjuvant (aluminium hydroxide 2 mg/mL).

The control product was sterile water for injection.

Administration (1 mL) was by the subcutaneous route behind the left shoulder blade on day 0 and behind the right shoulder blade on day 21 using standard aseptic technique.

2.3. Challenge

The four challenge isolates were heterologous strains to the vaccine antigens and have been used previously in validation studies. *L.icterohaemorrhagiae* was originally derived from American Tissue Culture Collection (ATCC) 43782 (isolated from human blood), *L. grippotyphosa* was originally from ATCC 23604 (isolated from water), *L. canicola* was originally from ATCC 23606 (isolated from blood of dog) and *L. bratislava* was originally from the National Institute for Public Health, Prague (isolated from hedgehog). Approximately $9 \times 10^7$ organisms/mL were given per dog, with 0.1 mL administered into each eye and 0.8 mL injected intra-peritoneally with procedures as previously described [11].

2.4. Observations and Samples

Blood samples were collected from each animal prior to test material or challenge administration on days 0, 21, 388 and at the end of the study – day 416; with additional samples collected on days 42 and 63 and then at 3, 6 and 9 months post vaccination to prepare sera which were examined for antibody presence. Blood samples intended for the detection of challenge organism were collected into sterile tubes before and 2, 3, 4, 5, 8 and 11 days post challenge.

Samples of urine were collected into sterile tubes with 1% bovine serum albumin from each animal prior to challenge and 3, 5, 8, 11, 14, 21 and 28 days post challenge. Urine collection was performed directly from the urinary bladder using a sterile needle and standard aseptic technique (cystocentesis).

Animals were humanely euthanased at the end of the study, and liver and kidney samples were collected.

Clinical observations were performed daily from day –2 until day 28, and from the day of challenge until the end of the study. Clinical observations involved an examination of each individual animal and included observing for clinical signs of disease as well as any injection site abnormalities. Observations were scored daily with a score of 1 for apathy, mild conjunctivitis, mild decrease in appetite, mild temperature increases and any other abnormal sign not listed; a score of 2 for jaundice, lethargy, moderate conjunctivitis, moderate decrease in appetite and moderate temperature increases, and a score of 3 for moribund status, severe conjunctivitis, anorexia and severe temperature increases.

2.5. Laboratory analysis

All sample analysis was conducted by personnel unaware (masked) of the allocation to treatment of individual animals. Sera for serology were used for testing of antibodies against the relevant *Leptospira* serovar using a micro-agglutination test [10]. Antibody titres were expressed as the maximum reciprocal dilution of serum at which antibodies were detected. Serum, urine and tissue samples intended for examination of presence of challenge organism were cultured using Korthof medium (Leptospira HiVeg Medium Base [Korthof Modified, M457] Hi-Media, with sterile rabbit serum) with 5-fluorouracil (Sigma) immediately after the sampling and assessed for the presence of organisms after both 2 and 4 weeks of cultivation [12].

2.6. Statistical analysis

A Biometrist from Zoetis was responsible for all data summaries and analyses (SAS Release 9.2, SAS Institute, Cary, NC). All hypothesis tests were conducted at the 0.05 level of significance (two-sided). For each of the four studies the statistical analysis was performed as described below.

Descriptive statistics for antibody titres against *Leptospira* including the geometric mean, minimum and maximum were calculated for each treatment and time point.

Clinical scores were calculated for each animal after challenge. Descriptive summary statistics of the scores data including the mean, median, minimum and maximum were calculated for each treatment. The ranks of the scores were analysed with a general linear mixed model, with a fixed effect of treatment and random effects of block and residual. Non-vaccinated animals were compared to vaccinated using contrasts across sex if the sex by treatment interaction was not significant or by sex if the sex by treatment interaction was significant.

The number of days of positive blood culture and positive urine culture were calculated for each animal. The number of days positive for presence of an organism in urine samples was analysed using a general linear mixed model with a fixed effect of treatment and the random effects of block (date of birth within gender) and residual. Least square means, standard errors, 95% confidence intervals, minimums and maximums were calculated for each treatment.
Table 1
Clinical signs following challenge with L. kirschneri serovar grippotyphosa or L. interrogans serovars canicola, bratislava or icterohaemorrhagiae for non-vaccinated dogs\(^a\). Results indicate the number of control dogs affected and maximum duration of clinical sign.

<table>
<thead>
<tr>
<th>Clinical sign</th>
<th>L. grippotyphosa</th>
<th>L. canicola</th>
<th>L. bratislava</th>
<th>L. icterohaemorrhagiae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apathy</td>
<td>1/6 (8 days)</td>
<td>1/6 (4 days)</td>
<td>2/6 (5 days)</td>
<td></td>
</tr>
<tr>
<td>Anorexia</td>
<td>1/6 (3 days)</td>
<td>2/6 (8 days)</td>
<td>2/6 (4 days)</td>
<td>2/6 (5 days)</td>
</tr>
<tr>
<td>Dehydration</td>
<td>1/6 (2 days)</td>
<td>2/6 (4 days)</td>
<td>2/6 (4 days)</td>
<td></td>
</tr>
<tr>
<td>Jaundice</td>
<td>1/5 (5 days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td>2/6 (7 days)</td>
<td></td>
<td></td>
<td>4/6 (12 days)</td>
</tr>
</tbody>
</table>

\(^a\) Only one vaccinated dog displayed any abnormal clinical signs, as outlined in the text.

Table 2
Mean, minimum and maximum antibody titres by treatment group and time period for each Leptospira study. Antibody titres were determined by micro-agglutination test (MAT).

<table>
<thead>
<tr>
<th>Leptospira study</th>
<th>Treatment</th>
<th>Sampling time point (day)</th>
<th>0 (V1)</th>
<th>21 (V2)</th>
<th>42 (3w)</th>
<th>63 (6w)</th>
<th>112 (3m)</th>
<th>203 (6m)</th>
<th>294 (9m)</th>
<th>388 (12m)</th>
<th>416</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. grippotyphosa</td>
<td>Vaccinate</td>
<td></td>
<td>2 (&lt;4, 4)</td>
<td>11 (4, 32)</td>
<td>57 (32, 128)</td>
<td>32 (16, 128)</td>
<td>25 (8, 64)</td>
<td>6 (4, 8)</td>
<td>4 (&lt;4, 8)</td>
<td>4 (&lt;4, 4)</td>
<td>406 (128, ≥512)</td>
</tr>
<tr>
<td>L. grippotyphosa</td>
<td>Non-vaccinate</td>
<td></td>
<td>2 (&lt;4, 4)</td>
<td>10 (&lt;4, 4)</td>
<td>57 (32, 256)</td>
<td>64 (16, 256)</td>
<td>36 (16, 128)</td>
<td>29 (8, 128)</td>
<td>23 (8, 128)</td>
<td>20 (8, 64)</td>
<td>512 (≥512)</td>
</tr>
<tr>
<td>L. canicola</td>
<td>Vaccinate</td>
<td></td>
<td>2 (&lt;4, 4)</td>
<td>2 (&lt;4, 4)</td>
<td>36 (16, 64)</td>
<td>40 (16, 128)</td>
<td>29 (8, 64)</td>
<td>8 (4, 16)</td>
<td>4 (&lt;4, 8)</td>
<td>4 (&lt;4, 16)</td>
<td>256 (64, ≥512)</td>
</tr>
<tr>
<td>L. canicola</td>
<td>Non-vaccinate</td>
<td></td>
<td>2 (&lt;4, 4)</td>
<td>2 (&lt;4, 4)</td>
<td>36 (16, 64)</td>
<td>40 (16, 128)</td>
<td>29 (8, 64)</td>
<td>8 (4, 16)</td>
<td>4 (&lt;4, 8)</td>
<td>4 (&lt;4, 16)</td>
<td>256 (64, ≥512)</td>
</tr>
<tr>
<td>L. bratislava</td>
<td>Vaccinate</td>
<td></td>
<td>2 (&lt;4, 4)</td>
<td>1 (4, 32)</td>
<td>45 (32, 128)</td>
<td>32 (16, 64)</td>
<td>25 (4, 128)</td>
<td>13 (4, 64)</td>
<td>10 (4, 32)</td>
<td>11 (4, 64)</td>
<td>128 (64, 256)</td>
</tr>
<tr>
<td>L. bratislava</td>
<td>Non-vaccinate</td>
<td></td>
<td>2 (&lt;4, 4)</td>
<td>2 (&lt;4, 4)</td>
<td>2 (&lt;4, 4)</td>
<td>2 (&lt;4, 4)</td>
<td>2 (&lt;4, 4)</td>
<td>2 (&lt;4, 4)</td>
<td>2 (&lt;4, 4)</td>
<td>102 (64, 256)</td>
<td></td>
</tr>
</tbody>
</table>

V1 = first vaccination, V2 = second vaccination, W = week (e.g., 3w) M = month (e.g., 3m, 6m).

Frequency distributions of positive/negative kidney or liver culture were calculated for each treatment. The number of samples positive for presence of an organism were analysed using a Fisher’s Exact test.

3. Results

In all four studies all of the vaccinated and control dogs completed their respective studies on day 416, 28 days following challenge.

3.1. Clinical observations

In each study there were no abnormal clinical observations recorded during the vaccination phase of the study and all animals remained in good health. In the L. icterohaemorrhagiae and canicola studies one vaccinated animal in each showed injection site swelling after vaccine administration. In both cases the swellings disappeared spontaneously four to five days post-administration.

All vaccinated animals remained in good health following their respective challenge procedures and had no abnormal clinical signs, except for one dog in the L. grippotyphosa study where jaundice was recorded for a 14 day period. This abnormal health event in a vaccinated dog was not considered challenge related as no Leptospira could be isolated from any samples collected. The mean and median clinical scores for vaccinated dogs following challenge with L. icterohaemorrhagiae, canicola and bratislava was therefore 0; following challenge with L. grippotyphosa vaccinated dogs had a mean score of 5 and median of 0 (range = 0–30). For control animals, the clinical signs, number of affected dogs, and the nature and duration of clinical signs for each study are shown in Table 1. A significant difference was found between the total clinical scores of vaccinated and control animals in the L. bratislava study only (P = 0.0165).

3.2. Serology

Antibody responses of vaccinated and control dogs in each study are shown in Table 2.

In all studies the control dogs remained seronegative until challenge, at which point they seroconverted. Measurable increases in antibody titres were seen in vaccinated dogs in each study 21 days after first vaccination, with further increases seen after second vaccination and peak values seen either three or six weeks later. In all studies the antibody titres in vaccinated dogs declined over the following months until after challenge when anamnestic responses were observed.

3.3. Leptospira isolation from blood

Table 3 shows the number of dogs with a positive Leptospira isolation, the mean number (and range) of days Leptospira were detected and the significance value between the control and vaccinated treatment groups for each challenge study. Following challenge with L. kirschneri serovar grippotyphosa, or L. interrogans serovars icterohaemorrhagiae or canicola, up to 2 (of 6 dogs per study) vaccinated dogs had positive blood cultures for up to 2 days duration. Despite this, in each study there were significant differences between vaccinated and control dogs in the mean number of days that challenge organisms were detected.

3.4. Leptospira isolation from urine

Table 4 shows the number of dogs with a positive Leptospira isolation, the mean number (and range) of days Leptospira were detected and the significance value between the control and vaccinated treatment groups for each challenge study. One vaccinated dog from the L. kirschneri serovar grippotyphosa challenge study had a single positive urine culture 14 days post-challenge. In each study there were significant differences between vaccinated and control dogs in the mean number of days that challenge organisms were detected.

3.5. Leptospira isolation from liver or kidney

Table 5 shows the number of dogs with a positive Leptospira isolation from kidney or liver and the significance value between the control and vaccinated treatment groups for each challenge study. In each study there were significant differences between treatment groups in the number of dogs from which challenge organisms were isolated from liver or kidney.
dog developed jaundice for reasons unrelated to challenge. The susceptibility of control dogs to infection had clearly diminished with age as only one study, with *L. interrogans* serovar *bratislava* challenge, showed significant differences between vaccinated and non-vaccinated dogs. The age related effect following experimental challenge appears to be a consistent observation in vaccine duration of immunity studies [12,18]. Vaccination with the DHPPi/L4R vaccine also prevented (*L. bratislava*) or reduced the prevalence of leptospiropenia in all four studies, with significant differences in the mean number of days that vaccinated and control dogs had positive *Leptospira* isolation from blood. The impact of vaccination with the DHPPi/L4R vaccine on urinary shedding and presence in kidney or liver was similar to that seen at onset of immunity [unpublished data]; only one vaccinated dog showed positive *Leptospira* isolation from urine and also gave positive culture from kidney and liver while all other vaccinated dogs were negative.

This study demonstrates that administration of the new *Leptospira* containing multivalent vaccine to six week old puppies induces immunity which after two vaccinations is still effective up to one year later against a challenge with *L. canicola, icteroahemorrhagiae, bratislava* or *grippotyphosa*.

### References


