Duration of immunity in dogs vaccinated against leptospirosis with a bivalent inactivated vaccine

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Abstract

Duration of immunity in dogs induced with current commercial inactivated leptospirosis vaccines and evaluated against experimental infection, to date, has hardly been documented. The purpose of the present work was to assess the duration of immunity in dogs that is attainable with a commercial inactivated bivalent leptospirosis vaccine. For this purpose, young dogs were vaccinated twice followed by challenge with either Leptospira interrogans serovar canicola or L. interrogans serovar icterohaemorrhagiae 5 weeks, 27 weeks or 56 weeks after the second vaccination. For assessment of the duration of immunity, titres of agglutinating serum antibodies were measured before and after challenge, and the effects of challenge on a variety of parameters were determined including reisolation of challenge organisms from blood, urine and kidney.

Both challenge strains induced a generalised infection in control dogs, the canicola strain being most virulent. From the results with different parameters it appeared that the two vaccinations induced a high rate of protection from generalised infection with canicola and icterohaemorrhagiae at 5, 27 and 56 weeks after the second vaccination. In addition, after 56 weeks, still a high level of immunity against renal infection with sv. canicola and, as a consequence, urinary shedding of sv. canicola bacteria, was demonstrated. It was, therefore, concluded that with this vaccine, using this vaccination schedule, a duration of immunity of 1 year can be attained against infection with both serovars.

Keywords: Canicola; Dog; Icterohaemorrhagiae; Leptospira interrogans; Vaccine

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1. Introduction

Since 1987, pathogenic strains of *Leptospira* can be assigned to one or more of seven officially recognised genospecies (Yasuda et al., 1987; Faine, 1994). One of these is the genospecies *Leptospira interrogans*. Historically, leptospirosis was recognised as a disease of dogs before it was known in any other animal species, including humans (Faine, 1994). *L. interrogans* serovar (sv.) canicola and sv. icterohaemorrhagiae are considered the most significant serovars in dogs worldwide. The dog and the rat are the maintenance hosts of sv. canicola and sv. icterohaemorrhagiae, respectively, which serve as reservoirs of infection. In dogs, sv. canicola is considered to be the most important causative agent of “Stuttgart disease”, an acute renal or gastrointestinal form of leptospirosis. Historically, sv. icterohaemorrhagiae has been associated with Weil’s disease, an acute haemorrhagic form of leptospirosis, but it can also cause a less acute hepatic syndrome or a uremic form with haemorrhagic enteritis (André-Fontaine and Ganière, 1990; Rentko et al., 1992; Faine, 1994). Comparison of several publications on clinical presentations of canine leptospirosis shows that no serovar is exclusively associated with any one clinical picture (Harkin and Gartrell, 1996). Although acute disease may result in death of the dog, those that survive may harbour the bacteria in their kidneys, becoming persistent shedders of the bacteria in the urine. Infections of humans with either sv. canicola or sv. icterohaemorrhagiae have been connected with contact with the urine of infected dogs. In 1995, a severe epidemic of leptospirosis in Nicaragua was most likely caused by exposure to flood waters contaminated by urine from dogs infected with sv. canicola (Trevejo et al., 1998).

In Europe and North America the prevalence of sv. canicola and—to a lesser degree—sv. icterohaemorrhagiae infection in dogs is believed to have decreased because of the widespread use of canine vaccines against these two serovars (Ferguson, 1994) and the prevalence of some other serovars in dogs in the United States and Canada is even thought to be underestimated (Sasaki, 2002). The former opinion is mainly based on results of serological surveys, in which titres of serogroup-specific agglutinating serum antibodies (predominantly IgM) were determined. With these antibodies mainly recent infections are demonstrated. However, nothing is known of the number of subclinically infected dogs or renal carriers of sv. canicola and sv. icterohaemorrhagiae. If, despite this uncertainty, the prevalence of sv. canicola and sv. icterohaemorrhagiae is indeed falling it can be anticipated that the infection pressure would rise again if vaccination against these two serovars were to be omitted.

Most commercial vaccines against canine leptospirosis are chemically inactivated whole cell vaccines. In the 1980s and 1990s the efficacy of the available commercial vaccines in preventing not only clinical leptospirosis, but also the renal carrier state in dogs and other animal species was extensively discussed, since in some publications this efficacy was questioned (Marshall, 1983; Wohl, 1996). More recently, questions have been raised about the duration of immunity of dog vaccines, including those against canine leptospirosis (Coyne et al., 2001). Based on immunoglobulin G (IgG) titres in dogs after two vaccinations with a commercial vaccine against sv. canicola and sv. icterohaemorrhagiae a duration of immunity of approximately 3 months was suggested (Hartman et al., 1984). However, in recent studies with cattle the paradigm that protective immunity against leptospirosis is primarily humoral was re-evaluated, and indeed significant cell-mediated immunity was
induced by vaccination (Ellis et al., 2000; Naiman et al., 2001). To our knowledge, there is as yet only one publication in which inoculation with leptospires was used to determine the duration of immunity in vaccinated dogs (Tronel et al., 1999). However, the authors claimed a duration of immunity of 10 months only against sv. canicola infection. Results of duration of immunity after sv. icterohaemorrhagiae infection were not included in this paper. The purpose of the present work was to assess the duration of immunity in dogs that is attainable with a commercial inactivated bivalent leptospirosis vaccine by vaccinating young dogs twice followed by challenge with either sv. canicola or sv. icterohaemorrhagiae 5, 27 or 56 weeks after the second vaccination.

2. Materials and methods

2.1. Animals

Healthy, specific pathogen-free Beagle dogs supplied by Harlan Nederland, Horst, The Netherlands, aged 9 ± 2 weeks and of both sexes were selected for these studies. Only dogs seronegative for sv. canicola and sv. icterohaemorrhagiae were admitted. With these dogs, age- and sex-matched treatment groups were formed (n = 6 per group). All housing systems used in these studies fully complied with the requirements of the Federation of European Laboratory Animal Science Associations (FELASA). The animal study was reviewed and approved by the Ethical Committee and conducted under the Dutch Animal Experimentation Law.

2.2. Treatments

Three separate vaccination-challenge experiments in dogs were performed. Basically, the treatments were similar in the three studies (referred to as studies 1–3). Per challenge type (sv. canicola or sv. icterohaemorrhagiae) two treatment groups were formed: a test group and a control group. In all three studies each test group was vaccinated twice, 4 weeks apart (at the ages of approximately 9 and 13 weeks, respectively), by subcutaneous injection with one dose of the commercial vaccine Nobivac® Lepto (Intervet International BV, Boxmeer, The Netherlands). This is a non-adjuvanted liquid vaccine containing inactivated whole cells of serogroup Canicola and serogroup Icterohaemorrhagiae. In studies 2 and 3, this vaccine was combined with the commercial vaccine Nobivac® DHPPi (Intervet International BV, Boxmeer, The Netherlands). This is a freeze-dried vaccine consisting of modified live canine distemper virus, canine adenovirus type 2, canine parvovirus and canine parainfluenza virus. For preparation of the combination vaccine the freeze-dried vaccine (one dose) was dissolved in the liquid vaccine (one dose). In study 1 the control groups remained untreated prior to the inoculation of leptospires. In studies 2 and 3 the control groups were vaccinated twice, 4 weeks apart (simultaneously with the test groups), through a subcutaneous injection with Nobivac® DHPPi dissolved in Nobivac® solvent. The essential difference between the three studies was the interval between the second vaccination and the inoculation of leptospires. In study 1 this interval was 5 weeks, in study 2 this was 27 weeks and in study 3 this was 56 weeks.
For the experimental infection of the dogs, strain Moulton of sv. canicola and strain CF1 of sv. icterohaemorrhagiae were used. Shortly before the dog challenge both strains were passaged through hamsters to maintain or promote virulence. The actual challenge inocula were prepared from live organisms reisolated from the kidneys of moribund hamsters and subsequently passaged twice in liquid EMJH medium (Johnson and Harris, 1967). Rabbit serum free of agglutinating antibodies against sv. canicola and sv. icterohaemorrhagiae was added to the EMJH medium up to a final concentration of 1% (v/v) to enhance growth of the leptospires. In each study the inoculation of leptospires into the dogs was done as follows. Per study one test and one control group were challenged with either of the two challenge organisms. Each dog was given an intraperitoneal injection of 2 ml of challenge inoculum. In addition, from the same inoculum, 0.25 ml was instilled into the ventral conjunctival sac of each eye, so that the total amount given per dog was 2.5 ml. The total challenge dose per dog was \((5–6) \times 10^8\) organisms of sv. canicola or \((1–2) \times 10^9\) organisms of sv. icterohaemorrhagiae.

2.3. Serological test

Blood samples for serology were taken shortly before the first and second vaccination and the challenge, and 4 weeks post-challenge. Serum was prepared according to standard procedures. Serogroup-specific, agglutinating serum antibodies against serogroup canicola or serogroup Icterohaemorrhagiae were detected with the microscopic agglutination test (MAT) using standard procedures (Terpstra, 1992; Faine, 1994). This test is the international “gold standard” test for diagnosis of a recent leptospiral infection in humans or animals (Terpstra, 1992; Faine, 1994). The strains used as reference antigens in the MAT were sv. canicola strain Moulton and sv. icterohaemorrhagiae strain CF1.

2.4. Clinical examination

Rectal temperature was measured to assess the effects of leptospiral challenge on the dogs. For this purpose, rectal temperature was measured from 1 or 2 days before challenge until 15 days after challenge. All dogs were observed daily for clinical symptoms post-challenge.

2.5. Haematology

The following tests were only performed in studies 2 and 3. Blood was taken pre-challenge and at 6, 13, 20 and 27 days post-challenge. Blood samples using EDTA as anticoagulant were tested for leukocyte count, differentiation of leukocytes and thrombocyte count. Differentiation of leukocytes included numbers and percentages of neutrophilic polymorphonuclear granulocytes, neutrophilic granulocytes with band-shaped nucleus, eosinophilic granulocytes, basophilic granulocytes, lymphocytes, lymphoblasts and monocytes. These tests were performed by the UKG lab (a clinical/chemical lab) of the Clinic for Companion Animals of the Veterinary Faculty, State University of Utrecht, Utrecht, The Netherlands, using an ABX Helios automatic analyzer.
2.6. Reisolation of challenge organisms

Blood samples were taken for blood culture pre-challenge (day 0) and at various times between days 0 and 28 post-challenge (Fig. 3). Immediately after blood sampling 0.5 ml of heparinised blood was added to 10 ml of liquid EMJH medium (containing 200 μg/ml 5-fluorouracil and 1% (v/v) seronegative rabbit serum). These cultures were incubated at 28–30°C, and observed every 2 weeks using dark-field microscopy for a total of 8 weeks. Per study, from the blood cultures positive for sv. canicola or sv. icterohaemorrhagiae 5–10 cultures were subcultured in EMJH medium, and the subcultures tested for identification at the serogroup/serovar level using the MAT. The difference from the MAT used for serology was that here serogroup/serovar-specific reference sera were used to identify reisolated bacteria.

Urine sampling by bladder puncture was performed on day 0 and then once a week until 4 weeks post-challenge. Urine cultures were made as described above for blood cultures. In studies 1 and 3 the remaining urine (>1 ml) was centrifuged (15 min, 10,000 × g) and the pellet was resuspended in 200 μl Tris–EDTA buffer and stored at −20°C until use for PCR testing (see below).

From one of the kidneys removed during necropsy 4 weeks post-challenge (see below) 1–2 g of cortex tissue was taken aseptically for culturing of leptospires. The tissue sample was put into 10 ml of phosphate-buffered saline (pH 7.2), and homogenised with an Ultraturrax® homogenizer (IKA-Werke, Staufen, Germany). One hundred-fold dilutions of kidney homogenate in EMJH medium were cultured and examined as described above.

2.7. PCR on urine

DNA isolation from urine samples was done with a commercial kit (High Pure PCR Template Preparation Kit, Boehringer Mannheim, Germany), using the manufacturer’s manual. A PCR was performed using the primers published by Gravekamp et al. (1993), with minor modifications. These primers were LEP secY F1 (5′-AAACAAA TGGTCGGAAGA-3′) and LEP secY R1 (5′-CTGAA TCGCTGTA TAAAAGTA-3′). This primer set amplifies the secY gene from leptospiral strains belonging to the pathogenic species \textit{L. interrogans}, \textit{Leptospira borgpetersenii}, \textit{Leptospira weilii}, \textit{Leptospira noguchii}, \textit{Leptospira santarosai}, and \textit{Leptospira meyeri} (amplified fragment 282 bp).

Gel electrophoresis for detection of the PCR products was done with standard procedures (Sambrook et al., 2001). The PCR samples that showed bands at the right position were considered positive. As positive control the amplicons of a dilution series of DNA isolated from a known number of leptospiral cells from both challenge strains was included.

2.8. Necropsy and histopathological examination

Four weeks after challenge, all dogs were euthanised after adequate sedation, immediately followed by post-mortem examination. Macroscopic examination was performed with particular attention to lungs, liver, kidneys and spleen. Tissue samples from liver, kidneys, spleen and conjunctiva were processed for histopathological examination following standard procedures and sections were stained with haematoxylin and eosin (HE) for
histopathological examination, and with Warthin–Starry (WS) silver stain for the detection of leptospires.

2.9. Statistical analysis

For analysis of normally distributed data the *t*-test was used, otherwise non-parametric tests were applied. Changes within groups over time were analysed with a paired *t*-test or Wilcoxon signed rank test, two-sided, and differences between vaccinated and control groups were analysed using a two-sample *t*-test or Mann–Whitney *U*-test, one-sided. The level of significance was set at 0.05.

3. Results

In Fig. 1 the MAT titres against sv. canicola (charts A–C) and sv. icterohaemorrhagiae (charts D–F) in the period prior to challenge and 4 weeks post-challenge are given for studies 1, 2 and 3, respectively. In all studies, but particularly in study 3 relatively low MAT titres
were found just prior to challenge. In all studies, 4 weeks after challenge with sv. canicola, the highest titres against sv. canicola were seen in the respective control groups as compared to the vaccinates. The same was observed after challenge with sv. icterohaemorrhagiae. In Fig. 2 the rectal temperatures after challenge with sv. canicola or sv. icterohaemorrhagiae in studies 1–3 (charts A–C, respectively) are shown. Challenge with sv. canicola generally induced a more evident increase in temperature in the controls than in the vaccinates, most prominently shown in study 1. The only striking effect after challenge with sv. icterohaemorrhagiae was the sharp decrease of the rectal temperature in the control group on day 5 post-challenge in study 1 (chart A). No evident clinical symptoms associated with canine leptospirosis were observed, except in study 1 where one dog from the control group challenged with sv. icterohaemorrhagiae obviously showed wasting. In addition, this dog was leptospiraemic on days 1 and 2 post-challenge and hypothermic on day 5 post-challenge (rectal temperature 36.7 °C).

Hardly any change in total leukocyte counts was demonstrated in any of the studies (data not shown), except for a statistically significant decrease of total leukocyte counts on day 6 in the control group challenged with sv. canicola in study 2. In addition, the control group was statistically significantly different from the vaccinated group. In study 2 a statistically significant increase in the percentage of monocytes between days −1 and 6 was observed in both control groups, but not in the vaccinates (data not shown). Thrombocytopenia (<150 × 10^9 thrombocytes per litre) was demonstrated in part of the control dogs. In study 2 each control group had one thrombocytopenic dog, in study 3 four dogs from the control group challenged with sv. canicola had thrombocytopenia.

An overview of the results of blood and urine culturing in studies 1–3 is given for the individual dogs in Fig. 3. Regarding infection with sv. canicola as well as sv. icterohaemorrhagiae, vaccination induced complete prevention of leptospiraemia in study 1 and reduction of the prevalence and the duration of leptospiraemia in studies 2 and 3 (charts A and B). In addition, based on the number of positive blood cultures per dog after challenge with sv. canicola the vaccinates in studies 2 and 3 were highly significantly different from the controls. After challenge with sv. icterohaemorrhagiae the difference in number of positive blood cultures per dog between vaccinates and controls in study 2 was highly significant as well. In study 3 this difference was close to the level of significance (P = 0.054). Based on the results of urine culturing after challenge with sv. canicola (chart C) or sv. icterohaemorrhagiae (chart D) a high rate of protection against kidney infection was demonstrated in the vaccinates.

At culturing of kidney homogenate for reisolation of leptospires 4 weeks after challenge with sv. canicola six out of six control dogs were positive in study 1. In studies 2 and 3 three out of six and four out of six control dogs were positive, respectively. In each study all vaccinates remained negative. After challenge with sv. icterohaemorrhagiae no positive kidney cultures were obtained in any of the three studies from any group (data not shown).

With the PCR on urine samples grossly the same results were found as with urine culturing, although at several time points the PCR yielded positive samples where culturing gave negative results. For example, in study 1 two control dogs challenged with sv. icterohaemorrhagiae had positive urine cultures on days 14 and 20 only (Fig. 3D), but appeared to be shedding on day 28 based on PCR (data not shown).
Fig. 2. Rectal temperatures in dogs after challenge with sv. canicola or sv. icterohaemorrhagiae, in study 1 (A), study 2 (B) and study 3 (C). Triangles indicate average values in groups challenged with sv. canicola, circles indicate average values in groups challenged with sv. icterohaemorrhagiae. Open symbols represent vaccinated groups, closed symbols represent control groups. Bars indicate standard deviations (1 S.D.). Asterisks indicate a statistically significant difference between the control group and the concomitant vaccinated group.
Macroscopic abnormalities possibly associated with canicola challenge were only detected in some controls in study 3. Histopathological evidence of interstitial nephritis attributable to infection with sv. canicola was found in study 1 in four out of six control dogs and none of the vaccinates, and again in study 3 in five out of six control dogs and none of the vaccinates.

4. Discussion

With the present studies we demonstrate for the first time a duration of immunity of 1 year after two vaccinations with a commercial canine leptospirosis vaccine. All vaccinated dogs were protected for 13 months from renal infection with sv. canicola, whereas 83% of the control dogs were shedding sv. canicola. Further evidence of this protection is provided by interstitial nephritis that was only found in the control group. The protective effects after 13 months against sv. icterohaemorrhagiae infection were demonstrated by a reduction of
the prevalence and duration of leptospiraemia in vaccinated dogs compared with control dogs.

Typical serological findings in the present studies were the relatively low or even undetectable levels of agglutinating serum antibodies at the time of challenge in vaccinated animals that were protected from leptospiral infection. Lack of association between vaccine-induced protective immunity and MAT titres against leptospiral infection in dogs has also been observed in studies elsewhere (Heath and Box, 1965; Broughton and Scarnell, 1985; Steger-Lieb et al., 1999). In other animal species this has been reported as well (Marshall et al., 1979; Bey and Johnson, 1983; Bolin et al., 1991). In general, most problematic is the absence of detectable agglutinating antibodies in dogs that are shown to be protected. After challenge, however, typically high MAT titres were measured, which in all three studies were higher in the control groups than in the vaccinates. The higher “acute phase” MAT titres after challenge in control dogs, which have been reported in earlier vaccination-challenge studies in dogs (Kerr and Marshall, 1974; Broughton and Scarnell, 1985; Tronel et al., 1999), suggest that in the vaccinates part of the challenge organisms was rapidly eliminated by either humoral immunity (other than agglutinating antibodies) or cell-mediated immunity. In the present studies no antibody ELISAs, as alternative tests to the MAT, were used to measure titres of anti-leptospiral IgM or IgG in response to both antigens in the vaccine. The IgM- and IgG-specific ELISAs at that time developed by the group of Hartman appeared to be less suitable to differentiate between antibodies against specific serogroups/serovars of pathogenic *Leptospira* species in dogs (Hartman et al., 1986). As far as we are aware, to date ELISAs detecting specific antibodies against either sv. canicola or sv. icterohaemorrhagiae in dog serum have not yet been published. Clearly, more attention has been and is still being paid to the development of sensitive, genus-specific ELISAs for early detection of leptospiral infection in humans and animals (Ribotta et al., 2000; Flannery et al., 2001). Apart from a humoral immune response that is not detectable with the MAT also cell-mediated immunity could have contributed to the protection shown in the present studies. Evidence for long-lasting cell-mediated immunity in cattle vaccinated with inactivated leptospirosis vaccines was published by Ellis et al. (2000) and Naiman et al. (2001). It can be expected that comparable cell-mediated responses to leptospiral antigens are present in other animal species, including dogs.

Although less pronounced than the culture results, rectal temperature, total leukocyte count, percentage monocytes and thrombocyte count also showed differences between vaccinates and controls challenged with sv. canicola. Polymorphonuclear (neutrophilic) leucocytosis and thrombocytopenia in varying gradations are known to occur during leptospiral infection (Rentko et al., 1992; Faine, 1994). Furthermore, in dogs monocytosis is not only associated with chronicity, but also with the acute phase of infectious diseases (Schalm et al., 1975). These haematological differences between the vaccinates and controls can, therefore, be ascribed to the generalised infection with sv. canicola in the controls. Challenge of control dogs with sv. icterohaemorrhagiae in three separate studies resulted in a reproducible leptospiraemia and—5 days after challenge at the age of 4 months—in hypothermia. Hypothermia can be induced by leptospiral toxins, for example peptidoglycans that cause injury of the vascular endothelium leading to the same pathophysiological events as known in endotoxic shock induced by LPS from Gram-negative bacteria (Dobrina et al., 1995; Cinco et al., 1996). In studies 1 and 2, in addition to leptospiraemia in all control
dogs, two out of six unvaccinated dogs became renal carriers of sv. icterohaemorrhagiae, whereas none of the vaccinates did.

In conclusion, the results described here imply that 5, 27 as well as 56 weeks after the second vaccination a high rate of protection from generalised infection with sv. canicola and subsequent urinary shedding was induced. In addition, 5, 27 as well as 56 weeks after the second vaccination a high rate of protection from generalised infection with sv. icterohaemorrhagiae was induced.

References


