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# A novel tetravalent *Leptospira* bacterin protects against infection and shedding following challenge in dogs

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**Recent evidence based on the current epidemiological situation suggests that vaccines against canine leptospirosis in Europe should be directed against infection with *Leptospira interrogans* (*sensu lato*) serogroups Canicola, Icterohaemorrhagiae, Grippityphosa and Australis. In the eight studies presented here, dogs were vaccinated with Nobivac L4 (MSD Animal Health), a new tetravalent inactivated vaccine containing antigen from four strains representing these four serogroups. The dogs were then challenged, together with unvaccinated control dogs, using heterologous strains from the same four serogroups. In four of the studies, pups without agglutinating antibodies against the four serogroups were vaccinated with Nobivac L4 vaccine. In a further four studies, Nobivac L4 vaccine was given 48 hours after administration of antiserum from vaccinated dogs designed to mimic the serological status of pups with maternally derived antibodies against these serogroups. In all eight studies, vaccine efficacy was assessed in terms of antibody response, clinical signs, fever, thrombocyte count, frequency of positive isolation of challenge organisms from blood, urine and kidney and frequency of interstitial nephritis. The results demonstrate that Nobivac L4 vaccine induces sterile immunity against leptospiraemia and renal infection with strains of serogroups Canicola, Icterohaemorrhagiae and Grippityphosa, and induces sterile immunity against leptospiraemia with a strain of serogroup Australis. Since sterile immunity was achieved in pups pretreated with antiserum as well, it can be concluded that this vaccine is also likely to be efficacious in the face of maternally derived antibodies in pups from the age of six weeks.**

## Introduction

As described in a recent review paper (Ellis 2010), commercial canine leptospirosis vaccines traditionally containing inactivated antigens of serogroups Canicola and Icterohaemorrhagiae have been available in Europe for more than 50 years. The recommendation in this review was to continue inclusion in the vaccine of antigen of serogroups Canicola and Icterohaemorrhagiae, plus the inclusion of antigen of serogroups Grippityphosa and Australis. The importance of continued vaccination of dogs against Canicola and Icterohaemorrhagiae was emphasised also in a review by Burr and coworkers in which strategies for diagnosis and prevention were outlined (Burr and others 2009). Whereas in the USA, currently, four canine vaccines with antigens of four serogroups (Canicola, icterohaemorrhagiae,

Grippityphosa and Pomona) are available, in Europe a number of traditional bivalent vaccines (Canicola, Icterohaemorrhagiae) and only one trivalent vaccine (Canicola, Icterohaemorrhagiae and Grippityphosa) are currently available.

Clear evidence for clinical leptospirosis in dogs caused by strains of serogroup Grippityphosa has been found in mainland Europe (Ellis 2010). In Germany, Grippityphosa has been reported as the predominant serogroup associated with canine leptospirosis (Geisen and others 2007). Several clinical and pathological presentations of this disease have been described in association with the serogroup Grippityphosa, including acute renal insufficiency, hepatitis and pulmonary haemorrhage syndrome (Bishop and others 1979, Brown and others 1996, Boutilier and others 2003, Greenlee and others 2004, Kohn and others 2010).

It has been demonstrated that strains of serogroup Australis (eg, serovar Bratislava) are responsible for reproductive, renal, hepatic and pulmonary disease in dogs (Thomas 1980, Van den Broek and others 1991, Adamus and others 1997, Adin and Cowgill 2000, Baumann and Flückiger 2001). Bratislava infections in dogs have been reported in a number of European countries, including UK, Germany, France, Switzerland, Austria and Italy (Thomas 1980, Van den Broek and others 1991, Kölbl and others 1995, Steger-Lieb and others 1999, Scanziani and others 2002, Geier-Doemling and others 2003, André-Fontaine 2006, Mastroilli and others 2007, Duchow and others 2009, Ellis 2010, Barmettler and others 2011). Currently however, there is no vaccine on the market with any efficacy claim in respect of Australis serovars.

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Apart from the need for protection against a broader range of serogroups than just *Canicola* and *Icterohaemorrhagiae*, other concerns about the efficacy of the vaccines have been raised and discussed, particularly the lack of protection from renal infection, renal carrier state and urinary shedding of leptospires (Marshall 1983, Thiermann 1983, Greene and Shotts 1990, Wohl 1996, Kalin and others 1999). Wohl (1996) concluded that vaccination at that time was effective in reducing the severity of *Icterohaemorrhagiae* and *Canicola* infection, but not in preventing the carrier state. In studies with different animal species (Huhn 1975, Goddard and others 1986), it was shown that a higher antigen dose was needed to achieve full protection from renal infection as opposed to protection solely against clinical disease. Urinary shedding is considered not only a risk for other animals but also a potential risk for humans, such as the pet owner and other family members, veterinarians and their personnel, and other people in close contact with dogs (Jansen and others 2005, Duchow and others 2009, Baer and others 2010, Houwers and others 2010, Rojas and others 2010). Prescott (2008) even suggested categorising canine leptospirosis as a reportable disease to increase awareness and improve registration of this disease by public health authorities. Strains of serogroups *Australis* and *Grippotyphosa* can cause clinical disease in man, with a number of human cases of these infections having been reported in recent decades within Europe (Cinco and others 1989, André-Fontaine and Ganière 1990, André-Fontaine and others 1992, Gerding and others 1997, Ciceroni and others 2000, Pischke and others 2010, Topic and others 2010).

This paper describes a series of studies which demonstrate the efficacy of Nobivac L4, a new tetravalent leptospirosis vaccine, against both leptospiroemia and renal infection with strains of these four serogroups and, by extension, against clinical disease.

## Materials and methods

Eight challenge studies were performed. Details of grouping, vaccination schedules and dosing are given in Table 1. In studies 1–4, immediate immunity was determined against challenge with four different *Leptospira* strains. In studies 5–8, immediate immunity was determined against the same four challenge strains, but prior to the first vaccination, the dogs received an intravenous dose of serum contain-

ing antibodies against the four vaccine antigens to mimic the presence of maternally derived antibodies (MDA) (see below).

## Dogs: husbandry and welfare

Six-week-old conventional beagle dogs without detectable agglutinating serum antibodies against *Leptospira* serogroups *Canicola*, *Icterohaemorrhagiae*, *Grippotyphosa* and *Australis* were provided by a commercial supplier. In each of the eight studies, treatment groups (with eight dogs per group) consisted of pups of both sexes and pups derived from different litters in order to prevent sex and litter effects interfering with treatment effects. The selected dogs were free of clinical abnormalities or disease prior to inclusion in these studies. Husbandry was the same in each study; during the first part of the study (prechallenge), the dogs were housed in the dog facilities of the supplier; at the age of eight weeks, the pups were weaned, and for the challenge phase of the study, the dogs were transferred to the animal facilities of MSD Animal Health, where, after being allowed to acclimatise for seven days, they were challenged at the age of 13 weeks. All housing systems used in these studies fully complied with the requirements of the Federation of European Laboratory Animal Science Associations. The animal studies described in this paper were conducted after prior written approval by the responsible ethics review committee, and thus, this work follows international, national and institutional guidelines for humane animal treatment, and complies with relevant legislation.

## Vaccines and vaccination

Nobivac L4 is a non-adjuvanted liquid vaccine for injection containing inactivated whole cells of *Leptospira interrogans* serogroup *Canicola* serovar *Portland-vere*, *L. interrogans* serogroup *Icterohaemorrhagiae* serovar *Copenhageni*, *L. kirschneri* serogroup *Grippotyphosa* serovar *Dadas* and *L. interrogans* serogroup *Australis* serovar *Bratislava*. In studies 1–4, in addition to the Nobivac L4 vaccine, two vaccines licenced in Europe were used: Nobivac DHPPi and Nobivac KC. Nobivac DHPPi is a freeze-dried, non-adjuvanted vaccine for injection consisting of live attenuated canine distemper virus, canine adenovirus type 2, canine parvovirus and canine parainfluenza virus. Nobivac KC is a freeze-dried, non-adjuvanted vaccine for intranasal administration consisting of live attenuated canine parainfluenza virus

TABLE 1: Experimental set-up (vaccination – challenge)

Study (dogs per group)	Treatment group	Antiserum admin. (48 hrs before 1st vacc.)	Vaccines and route	First vaccination		Second vaccination		Serogroup used for <i>Leptospira</i> challenge†
				Age (weeks)	Volume (ml)	Age (weeks)	Volume (ml)	
1 (8)	L4	-	L4*+DHPPi* sc KC† intranasal	6	1 0.4	10	1 -	<i>Canicola</i>
	Control	-	DHPPi sc KC intranasal	6	1 0.4	10	1 -	
2 (8)	L4	-	L4+DHPPi sc KC intranasal	6	1 0.4	10	1 -	<i>Icteroh.</i>
	Control	-	DHPPi sc KC intranasal	6	1 0.4	10	1 -	
3 (8)	L4	-	L4+DHPPi sc KC intranasal	6	1 0.4	10	1 -	<i>Gripp.</i>
	Control	-	DHPPi sc KC intranasal	6	1 0.4	10	1 -	
4 (8)	L4	-	L4+DHPPi sc KC intranasal	6	1 0.4	10	1 -	<i>Australis</i>
	Control	-	DHPPi sc KC intranasal	6	1 0.4	10	1 -	
5 (8)	L4	YES	L4 sc	6	1	10	1	<i>Canicola</i>
	Control	YES	-	-	-	-	-	
6 (8)	L4	YES	L4 sc	6	1	10	1	<i>Icteroh.</i>
	Control	YES	-	-	-	-	-	
7 (8)	L4	YES	L4 sc	6	1	10	1	<i>Gripp.</i>
	Control	YES	-	-	-	-	-	
8 (8)	L4	YES	L4 sc	6	1	10	1	<i>Australis</i>
	Control	YES	-	-	-	-	-	

\*In studies 1–4, in the groups designated 'L4' tetravalent leptospirosis vaccine mixed with DHPPi (live attenuated canine distemper virus, canine adenovirus type 2, canine parvovirus and canine parainfluenza virus) vaccine was used

†KC vaccine (live attenuated canine parainfluenza virus and *Bordetella bronchiseptica*) was only administered at six weeks of age

‡Bacterial cell concentrations (cells/ml) were (per study no.): no. 1,  $1.0 \times 10^9$ ; no. 2,  $1.0 \times 10^9$ ; no. 3,  $1.0 \times 10^9$ ; no. 4,  $9.5 \times 10^8$ ; no. 5,  $5.0 \times 10^8$ ; no. 6,  $1.0 \times 10^9$ ; no. 7,  $2.0 \times 10^9$ ; no. 8,  $1.0 \times 10^{10}$ . Challenge was done three weeks after the second vaccination

and *Bordetella bronchiseptica*. In studies 1–4, Nobivac DHPPi was used either simultaneously with Nobivac L4 by dissolving DHPPi vaccine in L4 vaccine prior to injection, or alone by the dissolving of DHPPi vaccine in Nobivac Solvent. In studies 1–4, Nobivac KC was dissolved in diluent for KC vaccine.

In all eight studies, the dogs were vaccinated twice, at the ages of 6 weeks and 10 weeks. In studies 5–8, the efficacy in the face of passive immunity was determined ('MDA+studies'). For these studies, a specific dose of antiserum was administered 48 hours prior to the first vaccination, as follows. Pooled antibody-positive serum was derived from beagle dogs that had been vaccinated three times with Nobivac L4 vaccine in an earlier study. The serum samples from these dogs were collected three weeks after the last vaccination. Agglutinating antibody titres in the pooled serum against serogroups Canicola, Icterohaemorrhagiae, Grippotyphosa and Australis were measured using the microscopic agglutination test (MAT) (see below), and were between 7 and 8 ( $\log_2$  value of reciprocal of dilution titre). All dogs in studies 5–8 received an intravenous dose of pooled serum 48 hours before the first vaccination according to the formula: 'Dose (ml) = (body weight (gram)  $\times$  0.07)/45'. The explanation of the formula is as follows. The circulating blood volume (ml) of pups approximately equals their body weight (gram)  $\times$  0.07. The range of targeted agglutinating antibody titres in the pups after serum administration was 1.5–2.5 ( $\log_2$  value). This range was based on results of an earlier study in which 10 pregnant bitches were vaccinated with a bivalent leptospirosis vaccine. Due to turbidity of sera, MAT titres  $<3$  ( $\log_2$  value) were not measurable in pups. However, based on the titres in the dams one week postpartum, and a reported half-life of dog immunoglobulin G (IgG) of eight days (Day 2007) estimated titres in six-week-old pups were (mean  $\pm$  sd): Canicola, 1.3  $\pm$  1.2; Icterohaemorrhagiae, 1.2  $\pm$  1.4.

Further details of the experimental set-up of the eight studies are described in Table 1. In studies 1–4, control groups received DHPPi vaccine (twice) and KC vaccine (once), and in studies 5–8, control groups were not vaccinated, so that in all studies the difference between test and control groups was the inclusion of Nobivac L4 in the test groups.

### Challenge strains of *Leptospira* and challenge

In studies 1 and 5, *L. interrogans* serogroup Canicola serovar Canicola strain Moulton was used as challenge strain, and in studies 2 and 6, *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhagen strain CF1 was used as challenge strain. Both strains had been received from WHO/FAO/OIE and National Leptospirosis Reference Centre, KIT Biomedical Research, Amsterdam, The Netherlands. In studies 3 and 7, *Leptospira kirschneri* serogroup Grippotyphosa serovar Bananal/Liangguang strain 11808 was used as challenge strain, and in studies 4 and 8, *L. interrogans* serogroup Australis serovar Bratislava strain As-05-101 was used as challenge strain. Both strains had been received from the United States Department of Agriculture, National Animal Diseases Center. For each of the four strains, the following procedure was followed. The actual challenge material was prepared from a culture stored in liquid nitrogen, and passaged twice in Ellinghausen-McCullough medium modified by Johnson and Harris (EMJH) (Faine 1994). The cultures stored in liquid nitrogen were isolates from experimentally infected animals: strains CF1 and 11808 were hamster kidney isolates, strain Moulton was a dog kidney isolate, and strain As-05-101 was a dog urine isolate.

In all studies, challenge was performed three weeks after the second vaccination using a combination of two challenge routes: intraperitoneal injection (2 ml in all studies except 4, where the volume was 3 ml) and conjunctival instillation (0.25 ml into the ventral conjunctival sac of each eye). Bacterial cell concentrations (cells/ml) in the challenge materials were determined by total direct microscopic count under dark-field microscopy. For bacterial cell concentrations of all challenge materials, see Table 1 (footnote †).

### Clinical observations before challenge

In the first part of the study (prechallenge), the dogs were observed on a daily basis for the presence of any clinical abnormalities.

### Clinical examination postchallenge

The dogs were examined regularly throughout each experiment, including measurement of body temperature after challenge using subcutaneous transponders.

### Blood and serum samples

Blood and serum samples were taken by jugular venepuncture at regular intervals during the experiment, for thrombocyte count, bacterial culture, PCR and leptospiral antibody assay.

### Antibody assay

The serum samples were analysed by the MAT against serogroups Canicola, Icterohaemorrhagiae, Grippotyphosa and Australis (Faine 1994). The microscopic agglutination titres were expressed as the reciprocals of the highest serum dilutions that induced 50 per cent agglutination.

### Thrombocyte count

Blood samples using EDTA as anticoagulant were used for assessment of thrombocyte counts after challenge using a Cell-Dyn 3500 cell counter (Abbott Laboratories, Abbott Park, Illinois, USA).

### Isolation of challenge organisms

EDTA blood or heparinised blood was used for isolation of challenge organisms from blood until day 21 postchallenge. For the same purpose, urine samples were collected at regular intervals by cystocentesis as described elsewhere (André-Fontaine and others 2003), and kidney tissue taken at necropsy 4 weeks postchallenge was sampled and processed as described previously (Klaasen and others 2003). Samples of blood, urine and kidney tissue homogenate were inoculated into EMJH medium and cultures were incubated and examined as described previously (Klaasen and others 2003).

### PCR

In studies 1–3, serum samples from days 0, 3 and 7, postchallenge, were examined for the presence of DNA from challenge organisms with a real-time PCR targeting the *secY* gene. This PCR is a validated assay and is suitable for detection of DNA from all pathogenic *Leptospira* species (Ahmed and others 2009). The PCR was performed by WHO/FAO/OIE and National Leptospirosis Reference Centre, KIT Biomedical Research, Amsterdam, The Netherlands. The PCR results were scored as positive, suspect or negative.

### Necropsy and histopathological examination

Dogs with severe clinical signs after challenge were humanely euthanased. Postmortem examination, including histopathological examination with special attention given to the detection of interstitial nephritis (Klaasen and others 2003), was undertaken in these cases.

### Criteria for a positive dog

The definition of a dog positive for infection was a dog with at least two positive samples of blood (by culture) or serum (by PCR) or urine/kidney (by culture) on different days, or a dog with challenge-induced nephritis or clinical or haematological evidence (thrombocytopenia) for leptospirosis. The definition of a dog positive for renal infection (carrier animal, persistent shedder) was a dog with at least one positive sample of urine/kidney from day 14 postchallenge onwards, or challenge-induced nephritis (demonstrated by histopathological examination).

The criterion of 'at least two positive samples' to qualify a dog as 'positive for infection' is based on the corresponding validity criterion described in the European Pharmacopoeia Monograph for Canine Leptospirosis Vaccine (inactivated) (0447). For qualification of a dog as 'positive for renal infection', however, the authors deviated from this criterion. This was because the presence of leptospirae in urine or kidney tissue from day 14 postchallenge onwards is considered evidence for an active renal infection based on previously reported scientific data on renal disease and urinary excretion pattern in leptospirosis (Faine 1998, Levett 2001).



## Statistical analysis

Statistical analysis was performed for the following parameters: (1) the number of dogs per treatment group complying with the criterion of 'positive for infection' (two-sided Fisher's exact test); (2) the number of dogs per treatment group complying with the criterion of 'positive for renal infection' (two-sided Fisher's exact test). The level of significance was set at  $P \leq 0.05$ .

## Results

### Clinical signs and mortality

Before the challenge, the following were observed. In total, four dogs (two from study 2, one from study 5 and one from study 6) were either found dead or were euthanased because of severe clinical signs in the period between the first and second vaccinations. Three of these cases occurred between days 7 and 16 after the first vaccination, one (in study 5) on day 14 after the second vaccination. None of the cases were considered to be related to vaccination, as was concluded based on (timing of) the following clinical signs and postmortem findings. Two pups in study 2 showed lethargy and respiratory signs, and were described as thin, from day 13 after the first vaccination onwards. One of these two pups was in the control group. At necropsy, (after death on day 16 after first vaccination) macroscopic and histopathologic findings led to a diagnosis of pneumonia. One pup in study 5 displayed growth retardation, wasting (described as thin) and weakness on day 14 after the second vaccination. This pup was humanely euthanased, and at necropsy, the only macroscopic finding was decolouration of the liver. Histopathological examination was impossible due to postmortem autolysis of the organs and tissues. From day 7 after the first vaccination, one pup in study 6 showed growth retardation and epistaxis. The pup was humanely euthanased and macroscopic examination revealed a thin animal with findings of pulmonary haemorrhage, bloody liquid in the pleural cavity, and a decoloured liver. Histopathological examination revealed pulmonary alveolar oedema, a diffuse, severe vacuolation in the hepatocytes, moderate cholestasis and lymphodepletion in the spleen. It was concluded that the clinical and pathological findings in these four pups must be ascribed to an intercurrent health problem which was sub-clinical until the time of the first recording of clinical signs, and which was possibly caused by either an infectious agent (study 1) or congenital anomalies and/or malnutrition in the lactation period (studies 1, 5 and 6). Additional proof against any relationship with vaccination in study 1 was that the morbidity and mortality occurred in one litter, which affected pups in both treatment groups. This suggests that the cause of these problems was related to prenatal or postnatal circum-

stances specific for that litter. General data of this dog colony provided by the supplier showed that in the period in which these studies were performed the average loss of pups between the ages of six weeks and nine weeks was 5–10 per cent. The four pups described above constitute 3.2 per cent of the total of 124 dogs used in these eight studies, which is even lower than the average loss observed in this dog colony.

After challenge, the following were observed. Clinical signs in all control dogs were only observed in studies 1 and 5 (Canicola challenge). In addition, in study 1, one control dog died as a result of the challenge, and in study 5, three control dogs were humanely euthanased because of severe clinical signs due to the challenge. The lowest degree and frequency of clinical signs in control dogs were observed in studies 3 and 7 (Grippotyphosa challenge) and studies 4 and 8 (Australis challenge). The clinical signs after Icterohaemorrhagiae challenge (studies 2 and 6) were less severe than after Canicola challenge, but more severe than after challenge with Grippotyphosa or Australis (results not shown). In study 7 (Grippotyphosa challenge), one control dog was humanely euthanased because of severe clinical signs due to the challenge. In all studies, the degree and frequency of fever correlated well with the severity and frequency of clinical signs (results not shown). In none of the eight studies were any postchallenge clinical signs seen in dogs which had received leptospirosis vaccine, except two dogs in study 1 (see Table 2): which showed transient signs of weight loss (thin animals) and lethargy postchallenge. Although in these two dogs all other infection parameters (culture and PCR results, thrombocytopenia, nephritis) were negative, it cannot be completely excluded that the signs were due to the challenge.

### Serology

In the four 'MDA+studies' (studies 5–8), detectable agglutinating serum antibodies against one or more of the four *Leptospira* serogroups were present prior to the first vaccination in 60 out of the 62 dogs that were available for the leptospira challenge. This demonstrated that the serum administration had resulted in an immunological status representative of MDA-positive pups. In all eight studies, antibody titres after two vaccinations with Nobivac L4 were measured that are typical for this type of vaccine (average per group between <1 and 7; results not shown). In studies 5–8, the average titres after two vaccinations were generally equal to or higher than the corresponding (serogroup-specific) average titres in studies 1–4, which suggests non-interference of pre-existing antibodies with the take of the vaccine. In all eight studies, after challenge, antibody titres were measured in control dogs that are representative of postinfection titres in unprotected dogs (average in control groups between 5 and 10; results not shown).

Table 2 Overview results cultures/PCR, clinical signs, thrombocytopenia and interstitial nephritis

Study (challenge)	Treatment group	Number of dogs/group with:				
		Positive blood cultures or PCR (serum) (at least two positive samples on at least two different days)	Positive urine and kidney cultures (at least one positive sample from day 14 post-challenge onwards)	Clinical signs	Thrombocytopenia	Interstitial nephritis
1 (Canicola)	L4	0/8	0/8	2/8*	0/8	0/8
	Control	6/8	7/8	8/8	7/8	7/8
2 (Icteroh.)	L4	0/7	0/7	0/7	0/7	0/7
	Control	5/7	6/7	2/7	0/7†	2/7
3 (Gripp.)	L4	0/8	0/8	0/8	0/8	0/8
	Control	1/8‡	6/8	2/8	3/8	0/8
4 (Australis)	L4	0/8	0/8	0/8	0/8	0/8
	Control	6/8	0/8	0/8	0/8	1/8
5 (Canicola)	L4	0/7	0/7	0/7	0/7	0/7
	Control	7/8	7/8	8/8	6/8	8/8
6 (Icteroh.)	L4	0/7	0/7	0/7	0/7	0/7
	Control	6/8	5/8	4/8	2/8	0/8
7 (Gripp.)	L4	0/8	0/8	0/8	0/8	0/8
	Control	7/8	2/8	1/8	4/8	3/8
8 (Australis)	L4	0/8	0/8	0/8	0/8	0/8
	Control	4/8	0/8	1/8	2/8	0/8

\*Two dogs only had mild, transient clinical signs, and no positive culture or PCR results, no thrombocytopenia and no interstitial nephritis; the mild clinical signs, however, complied with the criterion of 'dog positive for infection'

†Although none of the control dogs had thrombocytopenia ( $<200 \times 10^9$  per litre), a significant decrease in thrombocyte counts was found on day 3 postchallenge. (mean values on day 3: L4 group,  $674 \times 10^9$ ; control group,  $263 \times 10^9$ )

‡Five out of eight dogs had a positive PCR result on day 3 and a negative PCR result on day 7, and one out of eight dogs had a positive PCR result on days 3 and 7, so that (based on the definition of a positive dog) only one dog was positive

## Thrombocyte count

Thrombocytopenia (<200×10<sup>9</sup> thrombocytes per litre blood) was observed in dogs in the control groups in studies 1 and 5 (Canicola) and study 6 (Icterohaemorrhagiae) (see Table 2). Extremely low thrombocyte counts (<100×10<sup>9</sup> thrombocytes per litre blood) were strongly correlated with severe clinical signs or death (results not shown).

## Isolation of challenge organisms and PCR

The summarised results of all studies are shown in Tables 2 and 3 representing the effects of challenge with the four different strains in vaccinated and control dogs. Depending on the challenge strain used, leptospiromia was observed for a period of three to seven days mainly in the control groups. At group level, serum PCR results correlated well with blood culture results, and the combination of these two parameters demonstrated a high rate of protection from infection.

In Table 3 the proportion of dogs with infection or renal infection are shown per group. These proportions are based on the results shown in Table 2 and the criteria for a dog with infection and a dog with renal infection, respectively. Also included here is an indication of the statistical significance of the recorded differences between the vaccinated and control groups of each study. Challenge with all four strains resulted in the presence of infection in the control groups. Challenge with the strains of serogroups Canicola, Icterohaemorrhagiae and Grippotyphosa resulted in evidence of renal infection in the control groups. After challenge with the strain of serogroup Australis, no statistically significant difference in frequency of renal infection was demonstrated between the vaccinated group and the control group. This was due to minimal (study 4; 1/8) or no (study 8; 0/8) renal infection in the control groups.

## Necropsy and histopathological examination

During necropsy, macroscopic abnormalities were detected in studies 1 and 5 (Canicola challenge), mainly consisting of pale and flabby kidneys, and in one euthanased dog in study 7 (Grippotyphosa challenge) where an emaciated cadaver and a pale yellowish liver were observed. Histopathological examination showed a high prevalence of interstitial nephritis in studies 1 and 5 (Canicola; 7/8 and 8/8 control

dogs, respectively). Other studies in which interstitial nephritis in the control group was demonstrated were study 2 (Icterohaemorrhagiae; 2/7), study 4 (Australis; 1/8) and study 7 (Grippotyphosa; 3/8). None of the vaccinated dogs in any of the eight studies developed interstitial nephritis.

## Discussion

The studies presented here demonstrate that a new tetravalent leptospirosis vaccine induces sterile immunity against challenge shortly after vaccination, using representative strains of the four main serogroups causing canine leptospirosis in Europe (Ellis 2010). In other similar (unpublished) studies it has been shown that this immunity persists for at least one year following vaccination. In the studies described in this paper, we were able to reproduce, in the unvaccinated control dogs, a pattern of leptospiromia and urinary shedding of the challenge organisms that is characteristic for canine leptospirosis. Although clinical disease in the control dogs was only observed to any extent in the Canicola study this was not unexpected since similar findings have been published by other groups (Kerr and Marshall 1974, Broughton and Scarnell 1985, Schreiber and others 2005). It should be pointed out that our studies were deliberately designed to measure the ability of the vaccine to prevent infection and renal carriage, and thus, designed to minimise the risk of (per)acute mortality in control dogs which would have reduced the opportunity to gather adequate renal infection data as was seen in studies with pups by Minke and others (2009). Although the present studies do not, therefore, provide direct evidence of protection against clinical disease for all four serovars, it has been shown previously that preventing or significantly reducing infection, which is only achieved by vaccines with a sufficiently high concentration of protective antigen, can provide indirect evidence of protective immunity in the face of clinical disease (Huhn 1975, Goddard and others 1986, Minke and others 2009).

These studies allow some important conclusions concerning the efficacy of this vaccine. First, that the vaccine induces sterile immunity against infection and renal infection with strains of serogroups Canicola, Icterohaemorrhagiae and Grippotyphosa (none of the vaccinates showed a single positive blood, urine or kidney culture). Secondly, that the vaccine induces sterile immunity against infection with a strain of serogroup Australis, serovar Bratislava (none of the vaccinates showed a single positive blood culture). Additionally, in four studies, antibody-positive dog serum was administered to the dogs shortly before the first vaccination to mimic a natural MDA-positive status. Since sterile immunity was achieved in these four dog studies as well, it can be concluded that this vaccine is also likely to be efficacious in pups from the age of six weeks with typical levels of passive immunity.

In these studies, we were able to reproduce transient leptospiromia and urinary shedding of the challenge organisms in non-vaccinated control dogs for Canicola, Icterohaemorrhagiae and Grippotyphosa. The antishedding effect of the vaccine helps prevent transmission of the infection to other animals and to humans and, therefore, is an aid in preventing these zoonotic infections. Limited data from the literature are available describing the natural pathogenesis of canine infection with Bratislava (and related serovars within serogroup Australis) and the possible tissue tropisms of this group of serovars. One of the main reasons for this is presumably the difficulty of reisolation of these leptospires from organs of affected animals due to their more fastidious nature. However, cases were reported in which Bratislava bacteria persisted in the kidney of carrier dogs for at least three months (Ellis 2010). Also, reisolations of Bratislava strains from the genital tract of bitches (25 cases in the UK) and reisolations of serogroup Australis strains from clinically affected dogs in Switzerland were reported (Ellis 2010). In our challenge studies, the Bratislava strain was able to persist in the bloodstream of non-vaccinated control dogs for up to three days (studies 4 and 8), and to appear in the urine on day 3 postchallenge (study 4: 3/8; study 8: 1/8), and caused interstitial nephritis in one control dog (study 4), clinical signs (wasting) in one control dog, and thrombocytopenia in two other control dogs (study 8). Although no detectable renal infection was induced, the positive reisolation and other results obtained in these studies clearly demonstrate the dog-pathogenicity of Bratislava.

Table 3 Proportions of dogs with infection or renal infection\*

Study (challenge)	Treatment group	Number of dogs/group positive for:			
		Infection		Renal infection	
		Pos. dogs per group†	P	Pos. dogs per group‡	P
1 (Canicola)	L4	2/8 <sup>a</sup> §¶	0.0070	0/8 <sup>a</sup>	0.0002
	Control	8/8 <sup>b</sup>		8/8 <sup>b</sup>	
2 (Icteroh.)	L4	0/7 <sup>a</sup>	0.0006	0/7 <sup>a</sup>	0.0006
	Control	7/7 <sup>b</sup>		7/7 <sup>b</sup>	
3 (Gripp.)	L4	0/8 <sup>a</sup>	0.0014	0/8 <sup>a</sup>	0.0070
	Control	7/8 <sup>b</sup>		6/8 <sup>b</sup>	
4 (Australis)	L4	0/8 <sup>a</sup>	0.0070	0/8 <sup>a</sup>	1.0000
	Control	6/8 <sup>b</sup>		1/8 <sup>b</sup>	
5 (Canicola)	L4	0/7 <sup>a</sup>	0.0002	0/7 <sup>a</sup>	0.0002
	Control	8/8 <sup>b</sup>		8/8 <sup>b</sup>	
6 (Icteroh.)	L4	0/7 <sup>a</sup>	0.0070	0/7 <sup>a</sup>	0.0256
	Control	6/8 <sup>b</sup>		5/8 <sup>b</sup>	
7 (Gripp.)	L4	0/8 <sup>a</sup>	0.0002	0/8 <sup>a</sup>	0.0769
	Control	8/8 <sup>b</sup>		4/8 <sup>b</sup>	
8 (Australis)	L4	0/8 <sup>a</sup>	0.0070	0/8 <sup>a</sup>	1.0000
	Control	6/8 <sup>b</sup>		0/8 <sup>b</sup>	

\*These proportions were based on data from Table 2 and the criteria for infection/renal infection

†Criterion for 'dog positive for infection' is: a dog with at least two positive samples of blood or serum or urine/kidney on different days, or a dog with challenge-induced nephritis, or clinical or haematological evidence for leptospirosis

‡Criterion for 'dog positive for renal infection' is: a dog with at least one positive sample of urine/kidney from day postchallenge 14 onward, or challenge-induced nephritis (demonstrated by histopathological examination)

§The two different superscript letters (<sup>a</sup>, <sup>b</sup>) indicate a statistically significant difference between vaccinated and control group (two-sided Fisher's Exact test, P < 0.05)

¶Two dogs only had transient clinical signs, complying, however, with the criterion of 'dog positive for infection' (see also Table 2)

Based on the findings after challenge with strains of *Canicola*, *Icterohaemorrhagiae* and *Grippytyphosa*, and the complete absence of any Bratislava challenge effect in the vaccinated dogs, it can be concluded that this new tetravalent vaccine induces preventive immunity three weeks after a basic vaccination schedule of two doses against strains of serogroups *Canicola*, *Icterohaemorrhagiae*, *Grippytyphosa* and *Australis*.

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