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Efficacy of DAPPi$_2$-LR Canine Vaccine against Rabies and Leptospira Induced Clinical Signs, Mortality and Renal Carriage

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Abstract

Efficacy of the *Leptospira*l and rabies components of EURICAN DAPPi$_2$-LR, a canine combined vaccine, was demonstrated after a primary course of vaccination using one dose of EURICAN® DAPPi$_2$-L followed by one dose of EURICAN® DAPPi$_2$-LR. Challenges for leptospirosis (serovars Canicola and Icterohaemorrhagiae) were carried out in puppies 14 days after vaccination for the onset of immunity studies (OOI), in adults 13/14 months after vaccination for the duration of immunity studies (DOI), and for rabies in adults 13 months after vaccination. For OOIs, vaccination provided full clinical protection against severe *Leptospira* challenges and prevented renal carriage and detectable excretion of *Leptospira* in urine. For DOIs, clinical signs of leptospirosis were less severe in controls but were absent or mild and transient in vaccinated dogs, and urinary shedding was significantly reduced (frequency and duration). In addition, a single dose of EURICAN® DAPPi$_2$-LR induced rabies virus neutralizing antibodies in all dogs as early as 2 weeks post-vaccination and provided full protection in a rabies challenge performed after 13 months. Vaccine has a high efficacy profile with a quick onset of immunity as early as 2 weeks post vaccination, providing full protection against fatal leptospirosis caused by *L*. Canicola and *L*. Icterohaemorrhagiae and strong seroconversion against rabies. Long-term protection was also demonstrated by challenges performed 13/14 months post vaccination with reduction of mortality, clinical signs, infection, renal carriage, and bacterial excretion for *Leptospira* and protection against mortality and clinical signs for rabies.

Keywords: *Leptospira* Canicola; Icterohaemorrhagiae; Rabies; Vaccine; Onset and Duration Of Immunity; Challenge

Abstract

Leptospirosis is the most widespread zoonotic disease in the world, affecting a broad range of mammals. It is caused by infection with pathogenic *Leptospira* species [1]. Clinical signs associated with *Leptospira* infection in dogs range from subclinical infection to acute disease. Common features include anorexia, vomiting, lethargy, muscle pain, dehydration, jaundice, abdominal pain, diarrhoea, bloody urine, and death [1-6]. Acute renal failure is the predominant finding in symptomatic dogs, alone or associated with signs of hepatic injury [2, 3 6, 7]. Animals recovering from leptospirosis may become asymptomatic carriers harbouring virulent leptospires in the renal tubules for extended periods and shedding infectious leptospires into the environment, thus being a possible source of human leptospirosis [1,7]. Excretion in urine may be intermittent or continuous and the urinary concentration of bacteria may be high [1,4,5]. Infection of naïve animals results from contact of intact mucous membranes, such as ocular conjunctival, or abraded skin with infected urine or urine-contaminated soil, water, food, or bedding [1,4]. Humans are incidental hosts and usually become infected through occupational, recreational or domestic contact with the urine of carrier animals (such as the dog), either directly or via contaminated water or soil.

EURICAN® L is a whole cell, non-adjuvanted vaccine prepared from inactivated cultures of *Leptospira interrogans*,

serovars Canicola and Icterohaemorrhagiae. Historically, canine leptospirosis has mainly been associated with serovars Canicola and Icterohaemorrhagiae. Recently, the epidemiological situation in Europe was reviewed and the options for changes to the *Leptospira* components of dog vaccines in Europe were examined. It was concluded that the inclusion of serovars Icterohaemorrhagiae and Canicola is still recommended and that the spectrum should be extended [8]. It is unlikely that these two historical serovars will ever be eradicated given the number of unvaccinated dogs and the extensive rat population [3]. Therefore vaccination of the canine population to protect against clinical signs induced by serovars Canicola and Icterohaemorrhagiae should not be discontinued and should even more provide protection against renal shedding [9].

Rabies is a zoonotic disease, caused by the rabies virus, of the Lyssavirus genus, within the family Rhabdoviridae. Domestic dogs are the most common reservoir of the virus, with more than 95% of human deaths caused by dog-transmitted rabies. In a recent study, it is estimated that canine rabies causes worldwide approximately 59,000 human deaths annually [10], many of whom are children bitten by rabies-infected dogs (http://www.who.int/rabies/en). Rabies is a 100% vaccine-preventable disease. Elimination programs often revolve around mass dog vaccination campaigns, where at least 70% of the dog population should be covered in order to break the cycle of transmission in dogs, and to humans.

On one hand, in primo vaccinated dogs, it is generally accepted that monovalent rabies vaccines offer a better serological conversion rate than multivalents ones [11], increasing the success of passing the serological test with an antibody threshold at 0.5 IU/ ml. On the other hand, combined vaccines, in particular the LR combinations are likely to display negative interferences between components. This is a matter of concern in many countries where rabies remains prevalent or perceived as a potential risk, and where veterinarians, for practical reasons, are still using extensively such combination vaccine. A way to get around this concern is to optimize the antigen ratio between leptospirosis and rabies antigens, which is critical to maintain highest efficacy profile of the vaccine and reduce interferences. Unfortunately, very few data, if any, have been published to support this approach and confirm the efficacy of each antigen of such combined vaccines.

Here, we describe level of protection conferred by a bivalent LR formulation which was optimized and tested on dog that underwent a primary course of one dose of EURICAN® DAPPi$_2$-L followed by one dose of EURICAN® DAPPi$_2$-LR. Leptospiroses challenges (serovars Canicola and Icterohaemorrhagiae) were carried out according to European Pharmacopoeia 14 days and 13/14 months after vaccination and rabies challenge 13 months after vaccination. Results of these five clinical studies for short-term and long-term protection against leptospirosis and rabies are presented and discussed.

Materials and Methods

Experimental design

Institutional Animal Care and Use Committee approvals were obtained before conducting the studies. Once clinical signs appeared in control group, clinical examination was performed twice a day and any animals displaying serious and irreversible clinical signs that could lead to suffering were humanely euthanized based on predefined endpoints.

For leptospirosis, four separate vaccination-challenge studies were carried out to investigate the onset (OOI) and duration (DOI) of immunity provided by EURICAN® DAPPi$_2$-LR against *L. interrogans* serovar Canicola and L.interrogans serovar Icterohaemorrhagiae (referred to as studies 1-2 and 3-4 for OOI and DOI respectively, Table 1). Onset of immunity studies were validated according to European Pharmacopeia. In all studies, puppies received subcutaneously two injections of primary vaccination at the age of 7-9 weeks with EURICAN® DAPPi$_2$-L and at the age of 11-13 weeks with EURICAN® DAPPi$_2$-LR. Dogs from studies 1 and 3 were challenged with *Leptospira* Canicola at 2 weeks or 14 months post-vaccination, respectively. Similarly, dogs from studies 2 and 4 were challenged with *Leptospira* Icterohaemorrhagiae at 2 weeks or 13 months post-vaccination, respectively. Dogs were examined for clinical signs consistent with leptospirosis. Blood and urine samples were collected at regular intervals for *Leptospira* isolation, and a kidney sample was aseptically taken from each dog at necropsy.

Designation	Study	Group	# dogs	Challenge	
				Time after V2	Infecting agent
Onset of immunity	1 : OOI Lc	V	7	2 weeks	Lc
		C	6		
	2 : OOI Li	V	7	2 weeks	Li
		C	7		
Duration of immunity	3 : DOI Lc	V	8	14 months	Lc
		C	8		
	4 : DOI Li	V	12	13 months	Li
		C	8		
	5 : DOI rabies	V	25	13 months	Rabies
		C	10		

V=Vaccinated, C=Control, V2 = second vaccination Lc = *L. Interrogans* serovar Canicola, Li = *L. Interrogans* serovar Icterohaemorrhagiae

Table 1. Experimental design

Blood was also collected for serological (studies 1 and 2), haematological (studies 1, 2 and 3) and biochemical analy-

sis (studies 1, 2 and 3). At the end of the observation period or at euthanasia, dogs were necropsied and organs were removed for histological examination.

For rabies (referred to as study 5, Table 1), a vaccination-challenge experiment was carried out according to European Pharmacopeia to study the duration of immunity provided by EURICAN® DAPPi$_2$-LR. Puppies were vaccinated twice subcutaneously at the age of 8-9 weeks with EURICAN® DAPPi$_2$-L and at the age of 12-13 weeks with EURICAN® DAPPi2-LR (vaccinated group, n = 25), or again with EURICAN® DAPPi$_2$-L for puppies (control group, n = 10). Dogs were challenged with rabies 13 months after the second vaccination. All animals were confirmed to be seronegative for rabies and healthy at the beginning of the study. After challenge, dogs were examined for clinical signs consistent with rabies.

Vaccines

For the leptospirosis studies, production batches of EURICAN® L and EURICAN® LR (Merial, Lyon, France) vaccines were used as diluent to reconstitute a non-adjuvanted freeze-dried pellet, EURICAN® DAPPi$_2$, which is a vaccine containing live attenuated canine distemper virus, canine adenovirus type 2, canine parvovirus and canine parainfluenza type 2 virus. The experimental LR vaccine was used for the rabies study. EURICAN L® is a whole cell, non-adjuvanted vaccine prepared from inactivated cultures of *Leptospira* interrogans, serovars Canicola and Icterohaemorrhagiae. EURICAN® LR is an alumine hydroxyde adjuvanted vaccine containing inactivated whole cells of *Leptospira interrogans*, serovars Canicola and Icterohaemorrhagiae and inactivated rabies antigen. All batches of EURICAN® L or EURICAN® LR complied with the potency requirements of monograph 0447 (*Leptospira*) of the European Pharmacopoiea (2002). EURICAN® LR complied with the potency requirements of monograph 0451 (rabies) of the European Pharmacopoiea (1998).

Leptospirosis Challenges Studies

Animals

Beagle puppies aged from 7 to 9 weeks without detectable agglutinating antibodies against the principal serovars of pathogenic *Leptospira* were purchased from commercial suppliers and enrolled into each study (table 1).

Challenge strains

Leptospira interrogans serogroup and serovar Canicola, strain Moulton (National Veterinary Services Laboratory (NVSL), Ames, Iowa, USA) was used as challenge inoculum in studies 1 and 3. *Leptospira interrogans* serogroup and serovar Icterohaemorrhagiae, strain 193 (Pasteur Institute, Paris, France) was used as challenge inoculum in studies 2 and 4, respectively. The identity of all serovars was confirmed by the Pasteur Institute, Paris, France, using restriction fragment analysis.

Challenge Protocol

After an initial culture in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium, the strains were back-passaged twice in hamsters to prevent loss of virulence through adaptation to culture conditions. Moribund hamsters were humanely euthanised and their livers and kidneys or spleens were aseptically removed and homogenated in sterile saline. After harvest, the challenge strains were passaged once *in vitro* (EMJH medium) to allow a more precise quantification of the bacterial suspension. Each dog received 11 ml (studies 1, 2 and 3) or 13 ml (study 4) of challenge suspension with 0.5 ml instilled in the ventral conjunctival sac of each eye and the remainder administered intra-peritoneally. In studies 1 and 2, the total challenge dose per dog was around 3 x 10^9 organisms for *L*. Canicola and *L*. Icterohaemorrhagiae, respectively. In studies 3 and 4, each dog received around 6 x 10^9 organisms of *L*. Canicola and *L.* Icterohaemorrhagiae, respectively.

Clinical Examination

All animals were observed daily for 28 (studies 1 and 2) or 35 days (studies 3 and 4) after challenge for signs consistent with leptospirosis, including, apathy/prostration, dehydration, conjunctivitis, vomiting, diarrhoea, and cutaneo-mucosal signs. Signs were scored by use of a standardized protocol (Table 2). Rectal temperatures were taken on day 3 (study 1) or 5 (study 2) before challenge and recorded daily for 7 days (studies 1 and 2) or 14 days (studies 3 and 4) after challenge. Temperatures above 39.5°C were considered as hyperthermia. Dogs were weighed once a week until the end of the study. During the post-challenge clinical examination, any animals displaying serious and irreversible clinical signs that lead to suffering were humanely euthanized. For post-mortem examination, animals were necropsied and subjected to a macroscopic examination.

Laboratory Analyses

For serology, whole blood was collected before vaccination (day 0) then 3-5 days before and 28 days after challenge. Selected sera were tested for antibodies using microscopic agglutination titres (MAT) by the National Reference Centre for *Leptospira* (CNRL), Institut Pasteur, Paris, France, against *L. interrogans* serovar Canicola (study 1) or *L. interrogans* serovar Icterohaemorrhagiae (study 2). Since serology has limited value for evaluating the efficacy of vaccines against leptospirosis, sera from studies 3 and 4 were not tested. Antibody titers were expressed as the highest serum dilution that induced agglutination. The threshold of positivity was set at 100 (1/dil).

For haematology, blood samples were collected in heparin from dogs of studies 1 and 2 on day 3 or 5 before challenge and then on days 2, 3, 4, 5, 8 and 11 after challenge. In study 3, blood was collected on the day of challenge, then daily for

7 days after challenge. In studies 1 and 2, counts of platelets were performed by ORBIO Laboratory, Bron, France. In study 3, counts of white blood cells and platelets were performed using a MS-9 cell counter analyser (Melet Schloesing, France). In studies 1 and 2, platelet counts were compared to normal values provided by ORBIO laboratory. Haematology analyses were not performed for study4.

Clinical sign	Degree	Score
General appearance	Good	0
	Apathy	1
	Prostration	2
	Death	5
Conjunctivitis	Absence of sign	0
	Presence of conjonctivitis	1
Dehydration	Absent	0
	Slight	1
	Severe	2
Digestive signs, vomiting	Absence of signs	0
	Vomiting without abdominal pain	1
	Severe vomiting alone or severe vomiting with blood or severe vomiting with abdominal pain or severe vomiting with blood and abdominal pain or abdominal pain alone	2
Digestive signs, diarrhoea	Absence of signs	0
	Non bloody diarrhoea	1
	Bloody diarrhoea	2
Cutaneo-mucosal signs	Absence of signs	0
	Slight icterus visible only on ocular mucosa	1
	Obvious icterus (of all non-pigmented body surfaces) alone or obvious icterus with mucosa congestion or obvious icterus with haemorrhagic disorders (petechiae, suffusions, haemorrhages) or obvious icterus with mucosa congestion and haemorrhagic disorders	2

Table 2. Clinical scoring protocol for canine leptospirosis

For blood chemistry, whole blood samples were collected on heparin from dogs of studies 1 and 2 before challenge and then on days 2, 3, 4, 5, 8 and 11 after challenge. Sera were analyzed for urea, creatinine, alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) by ORBIO Laboratory, Bron, France. Urea, creatinine, ALT, AST and ALP were compared to normal values provided by the same laboratory. Biochemistry analyses were not performed for studies 3 and 4.

For the detection of leptospires in blood, in studies 1 and 2, blood samples were collected on heparin tubes before challenge (day -5/day -3) and on days 2, 3, 4, 5, 8, 11 after challenge. In studies 3 and 4, blood was collected before challenge (day 0/day -11), then daily for 7 days and on day 10 after challenge. Blood samples were immediately inoculated in liquid EMJH medium (1 ml of blood in 9 ml medium) and transferred to the laboratory. Serial 10-fold dilutions (up to 10-3) were made in the same media and incubated at 30°C. All the cultures were incubated for a maximum of 6 weeks and observed weekly for the presence of leptospires using dark field microscopy. For the detection of leptospires in urine and kidney, urine samples were collected before challenge (day -5/day -3) and on days 3, 5, 8, 11, 14, 21 and 28 after challenge for studies 1 and 2, or before challenge (day -11/day 0) and at 2, 3 and 5 weeks after challenge for studies 3 and 4. Urine samples were collected either spontaneously after subcutaneous injection of the diuretic furosemide (DIMAZON® , Intervet, France) (0.5 to 1 ml/kg bodyweight) (females) via urinary catheterisation (males) or by cystocentesis (males and females), or by direct bladder tap at the time of euthanasia. Samples were immediately inoculated in liquid EMJH medium added with 5 Fluorouracil at a concentration of 100 µg/ml (1 ml of blood in 9 ml medium) and transferred to the laboratory. Serial 10-fold dilutions (up to 10-3) were made in the same media. Samples from kidneys were collected aseptically. Approximately 5-8 grams of organ tissue were macerated into 10 ml of liquid EMJH medium added with 5 Fluorouracil at a concentration of 100µg/ml. Tissue debris was allowed to settle, and serial 10-fold dilutions were made as for urine samples. All the cultures (urine and kidney) were incubated at 30°C for a maximum of 6 weeks and observed weekly for the presence of leptospires using dark field microscopy. Samples of kidneys and livers were fixed with 10% buffered formalin and processed for microscopic examination following Hemalun-Eosine-Safran staining.

Analysis of the Results

Statistical analyses were carried out using SAS® software. The level of significance was set at P 0.005

Clinical Scores

Global Clinical Score (GCS) was derived from the assessments of each Individual Clinical Sign (ICS) measured on each puppy throughout the monitoring period (number of days of monitoring = ndm) by taking into account the effective length of the monitoring period in the case of the death of the puppy (effective number of days of monitoring = ndme).

For each puppy (i), GCS was defined as the sum of the ICS (k) over the day of measurement (j) and was computed as follows:

$$GCSwi = \sum_{j}\sum_{k} ICS_{j,k} \times \frac{ndm}{ndme_i}$$

The comparison of the vaccinated group to the control group was performed using the Mann-Whitney (Wilcoxon) test.

Leptospiremia and Leptospiruria

A dog was considered to have *Leptospira*emia or leptospiruria if at any time point at least one blood culture or urine

culture was positive. The frequency of dogs with *Leptospira*emia, leptospiruria or positive kidneys was compared between vaccinated and control dogs using Fisher exact test. The number of days with *Leptospira*emia or leptospiruria for each dog was compared between vaccinated and control dogs using Mann-Whitney test.

Haematological and Biochemical Data

In studies 1 and 2, for each dog and at each time point, a scoring system based on a 3-point scale was used: 0 if no parameter was outside the normal values range, 1 if 1 or 2 parameters were outside the normal values range and 2 if 2 or more parameters were outside the normal values range. A global biochemical and haematological score (GBS) was calculated for each dog corresponding to the sum of daily scores throughout the monitoring period. GBS were compared between vaccinated and control groups using Mann-Whitney test.

In study 3, platelet and WBC counts of vaccinated and control groups were compared using a mixed model with repeated measurements.

Rabies Challenge Study

Animals

Eight to 9-week-old beagle puppies were purchased from a commercial supplier.

Challenge strains

Rabies, strain New York (CDC, Laurenceville, USA) was used as challenge inocula in study 5.

Challenge protocol

All dogs were confirmed to be in good health and injected sub-cutaneously with ROBINUL-V ND (0.25 ml/5kg), before the anesthesia. Dogs were anesthesied with Ketamine (Imalgene 1000ND) and Xylazine (ROMPUNND) (0.05 ml/kg) of each) before being injected 13 months after the second vaccination with 0.5 ml per muscle crotaphyte of a dilution of the rabies challenge virus in order to obtain a titre of $10^{3.8}$ CCID50/ml. The rabies challenge virus titer was confirmed by backtitration after challenge at $10^{3.4}$ CCID50/ml.

Clinical examination

Dogs were monitored and scored daily during 95 days after challenge for rabies clinical signs (changes in behavior, sensitivity disorders, rabid cry, swallowing difficulties, salivation, loss of appetite, furious behavior, paresis, paralysis and death). Dogs were euthanised on day 95 (D95). Brains of all dogs were collected and tested for rabies virus detection by immunofluorescence [12] at the ANSES (French Agency for Food, Environmental and Occupational Health Safety, Nancy Laboratory for Rabies and Wildlife, France).

Laboratory analyses

Serum anti-rabies neutralizing antibody titers were measured on day 0 (injection with L2R combo), days 14, 28, 56, 84, 147, 175, 203, and day-2 before challenge, and on the day of euthanasia. Rapid fluorescent focus inhibition test (RFFIT) was used according to the technique described by [13], with a positive threshold of 0.5 IU/ml. In this assay, a 1.41 log10 titer is equivalent to 0.5 IU/ml of neutralizing antibodies.

Results

Leptospirosis

Humoral Responses to Vaccination and Challenge

At the start of studies 1 and 2 (OOI, Lc and Li respectively), all dogs were seronegative for serovars Canicola or Icterohaemorrhagiae, respectively. None of the serum of the vaccinated dogs had MAT detectable antibody titres against serovars Canicola or Icterohaemorrhagiae after vaccination. In study 1, antibody titres against serovar Canicola were detected 28 days after challenge in all 7 vaccinated dogs (range: 50-800) and in 5 out of 6 controls (range 100-200). In study 2, antibody titres against serovar Icterohaemorrhagiae were detected 28 days after challenge in 3 out of 7 vaccinated dogs (range: 100-400) and in 4 out of 7 controls (range: 50-400).

Clinical Signs

In studies 1 and 2 (OOI, Lc and Li respectively), all controls developed clinical signs (apathy/prostration, dehydration, vomiting, bloody diarrhoea and/or icterus), except one puppy which died suddently on day 4 after Canicola challenge, without any clinical signs in the preceding observations. All other controls were humanely euthanised between day 4 and day 11 post-challenge (DPC). In contrast, all vaccinates remained healthy and did not show clinical signs, except for four vaccinates which presented transient conjunctivitis probably due to the inoculation route of the challenge suspension, and one vaccinate which presented with mild dehydration from days 14 to 28 after Canicola challenge (Table 3). GCS were significantly higher in controls than vaccinates in studies 1 ($p = 0.001$) and 2 ($p < 0.001$).

As expected, clinical signs were less severe in control adults. In study 3 (DOI, Lc), dehydration, diarrhoea and/or prostration were observed in 5 out of 8 controls leading to the euthanasia of one animal on DPC7 after having shown bloody diarrhoea, severe dehydration and prostration. Conjonctivitis was observed in all controls and in 3 of 8 vaccinates. In study 4 (DOI, Li), one control was humanely euthanised on DPC5 after having shown bloody diarrhoea, vomiting, icterus and prostration. Conjonctivitis was observed in 7 out 8 controls and in 4 of 12 vaccinates. In contrast, 7 out of 8 vaccinated dogs in study 3 and 10 out of 12 vaccinated dogs in study 4 did not show any clinical signs during the observation period. One vaccinate in study 3 had petechiae on DPC13 and DPC15 and two vaccinates in study 4 were apathetic for 1 day only (DPC 1 and DPC8) (Table 3). GCS were significantly higher in controls than vaccinates in study 3 ($p = 0.002$) and in study 4 ($p < 0.001$).

	Onset of immunity				Duration of immunity			
	Canicola		Icterohaemorrhagiae		Canicola		Icterohaemorrhagiae	
Clinical signs	V	C	V	C	V	C	V	C
Apathy/prostration	0/7	5/6	0/7	6/7	0/8	1/8	2/12	1/8
Conjunctivitis	4/7	3/6	4/7	6/7	3/8	8/8	4/12	7/8
Dehydration	1/7	4/6	0/7	6/7	0/8	4/8	0/12	1/8
Vomiting	0/7	4/6	0/7	1/7	0/8	1/8	0/12	1/8
Diarrhoea	0/7	5/6	0/7	7/7	0/8	3/8	0/12	1/8
Icterus/petechiae	0/7	5/6	0/7	7/7	1/8	0/8	0/12	1/8
Global Clinical Score	**8.1**	**61.4**	**2.7**	**70.6**	**2.1**	**23.7**	**5.7**	**34.6**
p-value*	***0.001***		***<0.001***		***0.002***		***<0.001***	

Abbreviations: V= vaccinated; C= control.
* Mann-Whitney (Wilcoxon) test

Table 3. Clinical signs after challenge with *L*. Canicola and *L*. Icterohaemorrhagiae in vaccinated and control groups performed 14 days or 14/13 months after primary course of two doses of vaccine (Studies 1- 4)

Haematology and Biochemistry

Studies 1 and 2 (OOI, Lc and Li respectively): All controls became thrombocytopenic (number of platelets < 150 x 10^3/mm^3) for several days after challenge. In contrast, a transient thrombocytopenia was recorded in 4 out of 7 vaccinates in study 1 (Lc) from one to three days and in 2 out of 7 vaccinates in study 2 (Li) for a single day. All controls showed an increase in urea, creatinine, AST, ALT and/or ALP values. In contrast, the values of the vaccinates remained normal. The GBS was then significantly lower in vaccinates than in controls ($p < 0.001$ in both studies).

In study 3 (DOI, Lc), the platelet count was significantly lower in the controls than in the vaccinates over the 1 to 7 days post-challenge period ($p < 0.001$) and the WBC count was significantly higher in the controls than in the vaccinates over the 3 to 7 days post-challenge period ($p = 0.006$).

Isolation from the Blood

All blood samples were negative for leptospires before challenge.

Studies 1 and 2 (OOI, Lc and Li respectively): Leptospires could be isolated from the blood of all controls for at least 3 days following challenge. In contrast, none of the vaccinated dogs developed *Leptospira*emia, except one dog on a single day (day 3) after *L. canicola* challenge, indicating that infection was not established in any of the vaccinated dogs (Tables 4A and 4B). The frequency and duration of *Leptospira*emia were significantly lower in vaccinates than in controls in study 1 ($p = 0.005$ and $p = 0.001$, respectively) and in study 2 ($p < 0.001$ and $p < 0.001$, respectively).

Studies 3 and 4 (DOI, Lc and Li respectively): all animals (controls and vaccinates) developed *Leptospira*emia (Tables 4C and 4D). The duration of *Leptospira*emia was significantly shorter in vaccinates than in controls in studies 3 ($p=0.003$) and 4 ($p=0.009$).

Isolation of Leptospires from Urine and Kidney

Studies 1 and 2 (OOI, Lc and Li respectively): Leptospires were isolated from the kidneys of all control dogs. In study 1 (OOI, Lc), no urine sample could be collected from one control dog, and on the day of euthanasia (DPC4 or DPC5) from 2 other dogs, but leptospires were recovered from the urine samples from the 3 other control dogs. In study 2 (OOI, Li), all controls, except 1 dog euthanised on DPC5 (no urine sample was collected at that time), had a least one positive urine sample between DPC3 and DPC11. In contrast, none of the vaccinated dogs had positive urine or kidney cultures (Tables 4A and 4B). The frequency of dogs with at least one positive urine sample and the duration of leptospiruria were significantly lower in vaccinates than in controls in studies 1 ($p = 0.046$) and 2 ($p = 0.005$). The frequency of dogs with a positive kidney was significantly lower in vaccinates than in controls in both studies ($p < 0.001$).

Studies 3 and 4 (DOI, Lc and Li respectively): All controls from studies 3 and 4 shed leptospires in the urine, and the kidneys of 3 out of 8 controls in study 3 (DOI, Lc) and of all controls in study 4 (DOI, Li) were cultured positive for *Leptospira*. Leptospires were isolated from the urine of 1 out of 8 vaccinates in study 3 and from 3 out of 12 vaccinates in study 4. No vaccinates in study 3, and 4 vaccinates out of 12 in study 4 had positive kidneys (Tables 4C and 4D). The frequency of dogs with at least one positive urine sample and the duration of leptospiruria were significantly lower in vaccinates than in controls in study 3 ($p = 0.010$ and $p = 0.009$, respectively) and in study 4 ($p = 0.001$ and $p < 0.001$, respectively). The frequency of dogs with a positive kidney was significantly lower in vaccinates than in controls in study 4 ($p = 0.005$), but was not significant in study 3, due to the low number of control dogs with positive kidneys.

Necropsy and Histopathology

In studies 1 and 2 (OOI, Lc and Li respectively), renal lesions

Group	Dog No.	Days after challenge (*L.* Canicola)																
		Blood							Urine									Kidney
		-3	2	3	4	5	8	11	-3	3	5	8	11	14	21	28	28
Vaccinated	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	positive	**0/7**	**0/7**	**1/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**
Control	8	-	+	+	+	d	d	d	-	NS	NS	d	d	d	d	d	+
	9	-	+	+	+	+	+	d	-	-	NS	+	d	d	d	d	+
	10	-	+	+	+	d	d	d	-	-	NS	d	d	d	d	d	+
	11	-	+	+	+	+	d	d	-	-	NS	d	d	d	d	d	+
	12	-	+	+	+	+	+	d	-	+	+	+	d	d	d	d	+
	13	-	+	+	+	+	+	d	-	-	+	+	d	d	d	d	+
	positive	**0/6**	**6/6**	**6/6**	**6/6**	**4/4**	**3/3**	**NA**	**0/6**	**1/5**	**2/2**	**3/3**	**NA**	**NA**	**NA**	**NA**	**6/6**

+ culture positive
- culture negative
d : euthanasia/death
NA: not applicable
NS: no sample (empty bladder)

Table 4A. *Leptospira* isolation from blood, urine and kidney after *L. interrogans* serovar Canicola challenge (Study 1, OOI Lc). Dogs were challenged 14 days after a primary course of two doses of vaccine

Group	Dog No.	Days after challenge (*L.* Icterohaemorrhagiae)															
		Blood							Urine								Kidney
		-5	2	3	4	5	8	11	-5	3	5	8	11	14	21	28	28
Vaccinated	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	positive	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**	**0/7**
Control	8	-	+	+	+	+	-	d	-	-	+	+	d	d	d	d	+
	9	-	+	+	+	+	d	d	-	-	+	d	d	d	d	d	+
	10	-	-	+	+	+	+	d	-	-	+	+	d	d	d	d	+
	11	-	+	+	+	+	d	d	-	-	NS	d	d	d	d	d	+
	12	-	+	+	+	+	-	d	-	-	+	-	d	d	d	d	+
	13	-	+	+	+	-	-	-	-	+	+	+	+	d	d	d	+
	14	-	+	+	+	+	d	d	-	+	NS	d	d	d	d	d	+
	positive	**0/7**	**6/7**	**7/7**	**7/7**	**6/7**	**1/4**	**0/1**	**0/7**	**2/7**	**5/5**	**3/4**	**1/1**	**NA**	**NA**	**NA**	**7/7**

+ culture positive
- culture negative
d : euthanasia/death
NA: not applicable
NS: no sample (empty bladder)

Table 4B. *Leptospira* isolation from blood, urine and kidney after *L. interrogans* serovar Icterohaemorrhagiae challenge (Study 2, OOI Li). Dogs were challenged 14 days after a primary course of two doses of vaccine

Group	Dog No.	Days after challenge (*L.* Canicola)													
		Blood									Urine				Kidney
		0	1	2	3	4	5	6	7	10	0	16	21	35	35
Vaccinated	1	-	+	+	+	+	-	-	-	-	-	-	-	-	-
	2	-	+	+	+	-	-	-	-	-	-	-	-	-	-
	3	-	+	+	+	+	-	-	-	-	C	-	C	-	-
	4	-	+	+	+	+	-	-	-	-	-	-	-	-	-
	5	-	+	+	+	+	+	-	-	-	-	-	-	-	-
	6	-	+	+	+	+	-	-	-	-	-	-	-	-	-
	7	-	+	+	+	-	-	-	-	-	C	-	-	-	-
	8	-	+	+	+	+	+	-	-	-	-	+	+	-	-
	positive	**0/12**	**8/8**	**8/8**	**8/8**	**6/8**	**2/8**	**0/8**	**0/8**	**0/8**	**0/6**	**1/8**	**1/7**	**0/8**	**0/8**
Control	9	-	+	+	+	+	-	-	-	-	C	+	+	-	-
	10	-	+	+	+	+	+	+	+	-	-	+	+	-	-
	11	-	+	+	+	+	+	-	+	-	C	+	+	+	+
	12	-	+	+	+	+	+	-	+	-	-	+	+	-	-
	13	-	+	+	+	+	+	+	+	-	-	+	C	+	-
	14	-	+	+	+	+	-	+	+	-	C	+	C	+	+
	15	-	+	+	+	+	+	-	-	-	C	-	C	-	-
	16	-	+	+	+	+	+	+	+	-	-	+*	d	d	+
	positive	**0/8**	**8/8**	**8/8**	**8/8**	**8/8**	**6/8**	**4/8**	**6/8**	**0/8**	**0/4**	**7/8**	**4/4**	**3/7**	**3/8**

+ culture positive
- culture negative
d : euthanasia/death
NA= not applicable
*urine sample collected on DCP7 (day of death)

Table 4C. *Leptospira* isolation from blood, urine and kidney after *L. interrogans* serovar Canicola challenge (Study 3, DOI Lc). Dogs were challenged 14 months after a primary course of two doses of vaccine

Group	Dog No.	Days after challenge (*L.* Icterohaemorrhagiae)													
		Blood									Urine				Kidney
		-11	1	2	3	4	5	6	7	10	-11	14	21	35	35
Vaccinated	1	-	+	+	-	+	+	-	+	-	-	-	-	-	+
	2	-	-	-	-	+	+	-	+	+	-	-	-	-	+
	3	-	+	+	+	+	C	-	-	+	-	-	-	-	-
	4	-	+	-	-	+	+	+	+	-	-	-	-	-	-
	5	-	C	C	C	-	+	C	+	+	-	-	+	-	-
	6	-	+	+	+	+	-	C	+	-	-	-	-	-	-
	7	-	-	+	+	+	-	+	+	-	-	-	+	-	-
	8	-	-	+	+	+	+	-	+	+	-	-	+	-	+
	9	-	-	-	+	+	-	+	+	+	-	-	-	-	-
	10	-	+	+	+	+	-	+	+	+	-	-	-	-	-
	11	-	+	+	+	+	+	+	+	+	-	-	-	-	+
	12	-	-	+	+	-	+	+	+	+	-	-	-	-	-
	positive	**0/12**	**6/11**	**8/11**	**8/11**	**10/12**	**7/11**	**6/10**	**11/12**	**8/12**	**0/12**	**0/12**	**3/12**	**0/12**	**4/12**
Control	13	-	+	+	+	+	+	d	d	d	-	+*	d	d	+*
	14	-	+	+	+	+	+	+	+	+	-	+	+	-	+
	15	-	+	+	+	+	+	+	+	+	-	+	+	+	+
	16	-	+	+	+	+	+	+	C	+	-	+	+	+	+
	17	-	+	+	+	-	+	+	C	+	-	+	+	+	+
	18	-	+	+	+	+	+	+	+	+	-	+	+	+	+
	19	-	+	+	+	+	+	-	+	+	-	+	-	-	+
	20	-	+	+	+	+	+	+	C	+	-	+	+	+	+
	positive	**0/8**	**8/8**	**8/8**	**8/8**	**7/8**	**8/8**	**6/7**	**4/4**	**7/7**	**0/8**	**8/8**	**6/7**	**5/7**	**8/8**

+culture positive
- culture negative
* urine sample collected on DPC5 (day of euthanasia)
C=contaminated
d : euthanasia/death

Table 4D. *Leptospira* isolation from blood, urine and kidney after *L. interrogans* serovar Icterohaemorrhagiae challenge (Study 4, DOI Li). Dogs were challenged 13 months after a primary course of two doses of vaccine

were observed in all controls, ranging from rare and discrete clusters of inflammatory cells (lymphocytes, plasma cells) within cortical interstitium (study 2) to extensive mononuclear cell inflammatory infiltrates throughout the cortex, protein within tubules, tubular degeneration, glomerular senescence or cortical fibrosis (study 1). Hepatic lesions including discrete margination of neutrophils and lymphocytes into sinusoids, mild periportal, intraparenchymatous and centrolobular infiltration of lymphocytes and neutrophils, images of hepatocytic necrosis, dissociation of hepatocyte plates, or acute centrilobular degeneration were observed in all controls. No specific lesions were found in the vaccinated dogs. In studies 3 and 4 (DOI, Lc and Li respectively), macroscopic lesions were detected at necropsy in control dogs that were humanely euthanised. Gross findings included dehydration, icteric mucosa, liquid faecal material tinged with blood, petechiae or haemorrhagic lungs, jejunum, ileum, caecum, colon, liver and kidneys, and enlarged kidneys. When urine was present from these animals, it was tinged with blood up to severely haematuric. Apart from some reactive mesenteric lymph nodes and some enlarged spleens, control dogs that survived the experimental infection appeared normal on gross visual examination, as well as all vaccinated dogs. In study 4, kidney or liver lesions such as severe diffuse hemorrhagic nephritis, moderate to severe interstitial nephritis associated or not with tubular lesion of atrophy or pigmentary overload and an excess of bile pigment compatible with an hepatic cholestasis were observed in all controls. Vaccinated dogs had no lesions.

All results from the four studies are summarized in Table 5.

Rabies

Humoral Responses to Vaccination and Challenge

All controls animals remained negative for rabies (Figure 1), until the day of challenge with mean titers of 0.12 IU/ml (range: 0.06-0.18 IU/ml) at the time of vaccination and 0.07 IU/ml (range: 0.04-0.11 IU/ml) at the time of challenge. Vaccinated animals had a mean titer of 0.12 IU/ml at the time of vaccination (identical to control group, but in a range of 0.03-0.32 IU/ml, indicating that some dogs had traces of maternal antibodies). All vaccinated dogs seroconverted with antibody titres above the WHO positivity threshold value of 0.5 UI/ml, with a peak of antibody on day 28 post-vaccination (mean titer of 1.97 IU/ml, range 0.24-6.59 IU/ml). The mean antibody titres then decreased on days 84 (0.44, range 0.06-1.66 IU/ml), 175 (0.43 IU/ml, range 0.04-1.66 IU/ml), and became negative before challenge (0.18 IU/ml, range: 0.05-0.55). Mean specific antibody titers of the vaccinated group were higher than the mean values of the control group during the whole pre-challenge period, from 2 weeks post vaccination (1.16 IU/ml versus 0.10 IU/ml) and until the day of challenge (0.18 IU/ml versus 0.07 IU/ml).

Clinical signs

For rabies (table 6), 10 of 10 control dogs showed most of the signs of rabies. All the 25 vaccinated animals survived the rabies challenge. Some dogs showed non specific signs post-challenge, such as loss of appetite, or slightly modified habitus for 2 dogs, or very transient isolated swallowing

		Challenge 14 days after vaccination			Challenge 13 to 14 months after vaccination		
	Parameter	V	C	*p* value	V	C	*p* value
L. interrogans serovar Canicola	clinical signs	2/7	5/6**	0.001	3/8	8/8	0.002
	leptospiraemia	1*/7	6/6	0.005 (F) and 0.001 (D)	8/8	8/8	NS (F) and 0.003 (D)
	leptospiruria	0/7	3/6***	0.046 (F) and 0.046 (D)	1/8	7/8	0.010 (F) and 0.009 (D)
	positive kidney	0/7	6/6	< 0.001	0/8	3/8	NS
	kidney lesions	0/7	6/6	< 0.001			ND
L. interrogans serovar Icterohaemorrhagiae	clinical signs	4/7	7/7	<0.001	6/12	8/8	<0.001
	leptospiraemia	0/7	7/7	< 0.001 (F) and < 0.001 (D)	12/12	8/8	NS (F) and 0.009 (D)
	leptospiruria	0/7	6/7	0.005 (F) and 0.005 (D)	3/12	8/8	0.001 (F) and < 0.001 (D)
	positive kidney	0/7	7/7	< 0.001	4/12	8/8	0.005
	kidney lesions	0/7	7/7	< 0.001	0/12	8/8	ND

V: vaccinates, C:controls
D: duration
ND : not determined
F: frequency
NS: not significant
* positive on DPC3 only
** one control died on DPC4 before any clinical signs
*** no or only one urine sample tested for the 3 controls died on DPC 4 or 5

Table 5. Summary of Leptospiroses studies results (Studies 4-1)

disorders without other clinical signs, which could result from individual housing of the animal.

Immunofluorescence results

Brain tissue samples of all vaccinated dogs were found negative by specific immunofluorescence at the end of the study (table 6). In contrast, brain tissue samples from the ten control dogs were positive by immunofluorescence for rabies virus.

pies and adults remained healthy, without clinical signs, with only few animals developing mild and most often transient symptoms (dehydration, apathy, petechiae). The protective effect of the combined vaccine against severe clinical signs was demonstrated as soon as 14 days and lasted up to 13 to 14 months after a primary course of two doses of vaccine. Regarding laboratory data, hematological and biochemical abnormalities were observed only in unvaccinated control puppies and consisted of thrombocytopenia,

Group	Neutralizing rabies virus Antibodies seroconversion	Challenge resistance	Rabies diagnostics (immunofluorecence)
Group 1 (vaccinated with LR combo vaccine)	25/25	25/25	25/25: negative
Group 2 (vaccinated with L combo vaccine)	0/10	0/10	10/10 : positive

Table 6. Summary of rabies seroconversion, challenge result and virus isolation in test groups of dogs (Study 5)

Discussion

Since infection with leptospires can lead to an acute, often fatal disease [14], vaccination is recommended in dogs potentially exposed in order to protect them from signs of disease (and death in the worse case scenario) and to reduce significantly the shedding of virulent *Leptospira* and the zoonotic risk. Efficacy of vaccines can only be reliably evaluated through challenge studies with significant clinical signs of an acute disease and significant shedding in unvaccinated controls. Our challenge models were thus developped to induce typical clinical signs so as to allow demonstration of a complete protection against clinical disease and lesions associated to it. Only few studies in dogs have evaluated the efficacy of leptospirosis vaccines against a severe disease model following experimental challenge with *L*. Canicola [15-17] or L. Icterohaemorrhagiae [15,17,18]. In our challenge model, all control puppies of the onset of immunity studies showed symptoms of severe leptospirosis that led to euthanasia after *L*. Canicola or *L*. Icterohaemorrhagiae challenge. Symptoms observed in control puppies included dehydration, apathy, depression, vomiting, blood diarrhoea, icterus, and were characteristic of leptospirosis [2,3,5,6]. As expected, less severe clinical signs were observed when performing duration of immunity studies in adult controls. Most challenge models used to assess the duration of immunity induced by leptospirosis vaccines have failed to produce severe clinical signs in adult controls [19-22]. This difference may be related to the challenge strain, the number of passages of the challenge strain in vivo (hamster) before inoculation to dogs or the challenge dose. It is therefore remarkable that our challenge models were capable to induce in adult dogs clinical signs, sometimes leading to euthanasia (one dog each) after challenges performed with *L*. Canicola and with *L*. Icterohaemorrhagiae. This confirms observations from a previous experiment using a similar model [17]. Despite this severe challenge model, most vaccinated pup-

increase in urea, creatinine, transaminases (ALT, AST) and/or alkaline phosphatase activities, supporting the renal and hepatic dysfunctions. In contrast, none of the vaccinated puppies showed hematological or biochemical disorders. Hematological abnormalities were also recorded in adult controls, with a significant decrease in the platelet count and increase in leucocytes in controls compared to vaccinates. All these hepatic, renal, and haematological signs supported the polysystemic nature of leptospires infection and have commonly been reported after natural or experimental exposure [2,3,5,7,9,16-19,23]. Macroscopic examination was consistent with clinical signs and microscopic lesions of kidneys and/or livers were observed in all control puppies or adult controls (study 4), whereas no specific lesions were detected in vaccinates. These results support the protective effect of EURICAN® LR against renal and hepatic failures after challenge with *Leptospira*.

Several studies have documented that vaccine-associated *Leptospira* MAT titers are generally low and short-lived [5,17,19,20,24] and, they should not be used to predict resistance to *Leptospira* infection. Although immunity against leptospirosis is thought to be primarily humoral [1], we did not find any correlation between post-vaccination antibody titers and protection since all vaccinated dogs were protected after challenge whereas none of them seroconverted after vaccination, thereby confirming previous observations [17].

While leptospires were isolated from the blood of all control puppies of the onset of immunity studies for at least 3 consecutive days after challenge with *L*. Canicola and *L*. Icterohaemorrhagiae, none of the vaccinated puppies became *Leptospira*emic, except one. One vaccinated puppy was detected positive on a single occasion on day 3 after challenge with *L*. Canicola without showing any clinical signs, urinary shedding, positive kidney, hematological or biochemical abnormalities, suggesting that the leptospiremia

was very transient. Due to the virulence of challenge strains, all adult controls of the duration of immunity studies but also vaccinated dogs were detected positive for several days when the challenge was performed 13 or 14 months after primary vaccination. However the duration of *Leptospira*emia was significantly reduced in vaccinates after both *L*. Canicola and *L*. Icterohaemorrhagiae challenges. The absence of full protection against *Leptospira*emia was also reported by others in duration of immunity studies, with either a challenge dose 67 times lower (9×10^7 organsims/dog) [21] than the one used in our challenge model or in a challenge model where 50% only of adult control dogs became *Leptospira*emic [20].

Besides protecting against clinical signs, vaccination should prevent renal colonization and urine shedding, which is characteristic of a carrier or maintenance animal host. The renal carrier state is central to the persistence and epidemiology of leptospirosis [4]. In the onset of immunity studies, 100% of control puppies but none of vaccinated puppies had urine or kidney samples positive for *Leptospira* isolation after *L*. Canicola and *L*. Icterohaemorrhagiae challenges. A complete protection against renal carriage was more difficult to achieve in long-term studies, probably due to the severe challenge conditions in face of fading immunity. We found some discrepancies between urine and kidney isolation, this beeing also reported in previous observations [17]. This may be related to the presence of specific inhibiting enzymes from kidney cells [25], high urine osmolarity and pH [26], and/or the intermittent shedding of leptospires [26]. Nevertheless, the vaccine was able to provide a significant reduction of carriage/shedding under such conditions: urine samples were detected positive after L. Canicola and *L.* Icterohaemorrhagiae challenges in 87.5% and 100% of adult controls, respectively, whereas urine shedding was detected in only 12.5% and 25% of vaccinated adults, respectively. The urinary shedding, in terms of frequency of dogs with leptospiruria and duration of leptospiruria, was significantly reduced in long-term studies after challenges with both serovars. Kidney samples were positive after L. Canicola and *L*. Icterohaemorrhagiae challenges in 38% and 100% of adult controls, respectively and in 0% and 33% of vaccinated adults, respectively. The difference between vaccinated and controls was significant after *L*. Icterohaemorrhagiae challenge. The difference was not significant after *L*. Canicola challenge due to the too low number of positive kidney samples in adult controls. It should be reminded that the challenge dose that we used was probably much higher than the one observed during a natural exposure, suggesting that the protection against renal carriage might be almost complete in the field. Indeed, contrasting results have been published in the literature about the protection against the establishment of a renal carrier stage, with a lack of protective effect against urinary shedding for some vaccines evaluated shortly after primo-vaccination [9]. A significant long-term protection against urinary shedding and renal infection has been reported in less severe challenge models [20-22], with some urine samples detected positive for *Leptospira* in a few vaccinated dogs [20,22].

Rabies study shows serological data and challenge results 13 months after one dose of primary vaccination with the EURICAN® DAPPi$_2$-LR vaccine. The rabies challenge was validated, as 100% of control dogs showed most of the expected typical signs of rabies and had to be euthanised. Post mortem examinations of brain tissues confirmed the infection. Rabies antigen was detected by immunofluorescence test from brains of all animals from control group. In contrast, all the 25 vaccinated dogs resisted rabies challenge and were protected. Tissus collected from the vaccinates which survived after challenge were found negative, confirming that they were free of virus and protected. As expected, serological results confirmed the immunogenicity of the vaccine, as 100% of the dogs developed rabies antibody titers above 0.5 IU/ ml after vaccination. This is not necessarily obvious for combination vaccine, as it was previously shown that *Leptospira*l components may strongly interfere with the raise of antibody titers [11]. In addition, in the field, in primovaccinated dogs, monovalent vaccines showed a better serological conversion rate than multivalent ones [11]. Interestingly, the EURICAN® DAPPi$_2$-LR vaccine induced also an early seroconversion 15 days post-vaccination against rabies with a peak observed 29 days post-vaccination, indicating a quick onset of immunity. A rapid decrease of rabies antibodies was observed and confirms the observation made by Cliquet [11]. Despite the absence or low levels of antibibodies specific of rabies (below the WHO positivity threshold value of 0.5 UI/ml), all dogs resisted to rabies challenge 13 months after a single vaccination. These results suggest that quality of the priming and the initial seroconversion level are crucial for protection. Absence of antibody at the time of challenge in such correctly vaccinated animals does not mean absence of protection, as cell mediated response may also play an important role. In summary, these results demonstrate that a single injection with the EURICAN® DAPPi$_2$-LR vaccine induced rabies virus neutralizing antibodies of at least 0.5 IU/ ml in all dogs from 2 weeks post-vaccination and that those dogs were protected more than one year after vaccination against a virulent rabies challenge.

EURICAN® DAPPi$_2$-L was previously shown to provide a rapid onset of immunity and a long-lasting protection of at least 14 months against serovars Icterohaemorrhagiae and Canicola. The vaccine was able to prevent clinical disease and renal carrier state [17]. The antigen ratio between leptospirosis and rabies antigens of EURICAN® LR vaccine has been optimized, allowing a reduction of 2/3 of total amount of protein per dose compared to historical LEPTORABISIN®. Here, we show that a primary course of one dose of EURICAN® DAPPi$_2$-L followed by one dose of EURICAN® DAPPi$_2$-LR provided a quick onset of immunity as early as 2 weeks post vaccination with full protection against fatal leptospirosis caused by *L*. Canicola and *L*. Icterohaemorrhagiae and with strong seroconversion against rabies. Long-term protection against leptospirosis and rabies was also demonstrated by challenge 13/14 months post-vaccination, with reduction of mortality, clinical signs, infection, renal carriage, and bacterial excretion for *Leptospira* and protection against mortality and clinical signs for all dogs challenged with rabies. These results

demonstrate that the LR formulation of the tested vaccine is optimal, providing good protection against *Leptospira* infection without hampering the quality of rabies protection. This combined vaccine should help veterinarians to prevent clinical disease in dogs and limit the zoonotic risk and transmission of leptospirosis and rabies between animals.

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Conflict Of Interest

Most authors of this paper are employees of Merial, the manufacturer of EURICAN® DAPPi$_2$-L and EURICAN® DAPPi$_2$-LR. A.L. Guiot is an external scientific writer, she has no competing interest.

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