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1 **Onset and duration of protective immunity against clinical disease and renal carriage in**  
2 **dogs provided by a bi-valent inactivated leptospirosis vaccine**

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10

11 **Abstract**

12

13 Protection against clinical disease and prevention of the renal carrier state remain the key  
14 objectives of vaccination against leptospirosis in the dog. In the present paper, groups of dogs  
15 were vaccinated twice with a commercial bacterin (EURICAN<sup>®</sup> L) containing *Leptospira*  
16 *interrogans* serovars icterohaemorrhagiae and canicola and challenged with heterologous  
17 representatives of both serovars at 2 weeks (onset of immunity) or 14 months (duration of  
18 immunity) after the second vaccination. Control dogs were not vaccinated against  
19 leptospirosis and kept with the vaccinated dogs. The challenges, irrespective of the serovar,  
20 reliably produced clinical signs consistent with *leptospira* infection in the control pups with  
21 up to 60 % mortality. As expected clinical disease in the adult controls was less severe, but we  
22 were able to induce morbidity and mortality as well. Under these extreme challenge  
23 conditions, clinical signs in the vaccinated dogs were rare, and when observed, mild and  
24 transient in nature.

25 Following experimental infection, 100% of the control pups and 83% of the adult controls  
26 became renal carriers. Despite the heavy challenges, none of the 18 vaccinated puppies (onset  
27 of immunity studies) and only two out of the 16 vaccinated adult dogs (duration of immunity  
28 studies) developed a renal carrier state. These results show that a primary course of two doses  
29 of EURICAN<sup>®</sup> L provided quick onset and long-term protection against both clinical  
30 leptospirosis and the renal carrier stage. This vaccine should provide veterinarians with a  
31 powerful tool to prevent clinical disease in dogs and zoonotic transmission of leptospirosis to  
32 humans.

33

34 <sup>®</sup>*EURICAN* is a registered trademark of Merial in France and elsewhere

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36 **Keywords:** *Leptospira interrogans* serovars canicola and icterohaemorrhagiae, bacterin,  
37 vaccine, clinical leptospirosis, renal carriage, onset and duration of immunity

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## 1. Introduction

Leptospirosis is an important zoonosis of worldwide distribution caused by infection with spirochaetes belonging to the pathogenic species of *Leptospira*. Infection typically results from direct or indirect contact with urine of infected animals. The clinical signs associated with *Leptospira* infection range from subclinical to acute disease characterized by anorexia, vomiting, lethargy, muscle pain, dehydration, jaundice, abdominal pain, diarrhoea, bloody urine, and death. Renal failure is the predominant finding in symptomatic dogs, with a small percentage also showing evidence of liver disease (Greene, 1998, review; Boutilier and Schulman, 2003). Clinically recovered dogs frequently become asymptomatic renal carriers, and as such can be an important source of human leptospirosis (Center for Disease Control 1972, Trevejo et al., 1998). *Leptospira (L) interrogans* serovars icterohaemorrhagiae and canicola are the two serovars traditionally associated with disease in dogs (Hartman, 1984, Trevejo et al, 1998), but new serovars play an increasingly important role (Scanziani et al, 2002, Ward et al, 2004; Moore et al, 2006; Geisen et al, 2007; Stokes et al, 2007). Hence, leptospirosis is now recognised as an important re-emerging disease in dogs (Bolin, 1996; Ward et al., 2002). While short-term clinical protection has been demonstrated experimentally in dogs after vaccination with several vaccines (Huhn et al, 1975; Andre-Fontaine et al, 2003; Klaasen et al, 2003; Schreiber et al, 2005a, Schreiber et al, 2005b), there is much debate on whether leptospirosis vaccines protect against the renal carrier state (Andre Fontaine et al, 2003) or provide long term immunity (Cohne et al., 2001). As far as we know, we were the first to demonstrate 10 months duration of immunity against *L. interrogans* serovar canicola provided by a classical bacterin (Tronel et al, 1999). Since then only one paper has been published demonstrating duration of immunity of 13 months for a commercial bacterin (Klaasen et al. 2003). In the current study, we confirm and extend our previous observations and demonstrate that two doses of EURICAN<sup>®</sup> L provide both rapid onset and duration of protective immunity of at least 14 months against both serovars icterohaemorrhagiae and canicola. The vaccine was evaluated for protection against clinical disease and prevention of the renal carrier state.

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## 70 2. Materials and methods

71

### 72 2.1 Experimental design

73

74 Four separate vaccination-challenge experiments, including 74 puppies, were performed to  
75 study onset and duration of immunity provided by EURICAN L (referred to as studies 1-4,  
76 Table 1). Study 1 contained nine vaccinates and eight controls, study 2 nine vaccinates and ten  
77 controls, study 3 seven vaccinates and eight controls and study 4 nine vaccinates and ten  
78 controls (Table 1). Institutional Animal Care and Use Committee approvals were obtained  
79 before conducting the studies. In all studies, puppies were vaccinated twice subcutaneously, 4  
80 weeks apart. Puppies were 8-9 weeks of age at the time of first vaccination. Dogs from studies  
81 1 and 3 were challenged with *L. interrogans* serovar canicola at 2 weeks and 14 months,  
82 respectively, after the primary vaccination program of two doses. Dogs from studies 2 and 4  
83 were challenged with *L. interrogans* serovar icterohaemorrhagiae at 2 weeks and 14 months,  
84 respectively, after the second vaccination of the primary vaccination course. Because it is  
85 difficult to induce clinical leptospirosis in adult dogs, two 2-4 months-old pups were added to  
86 studies 3 and 4 at the time of challenge to assess the severity of the infection. Following  
87 challenge, dogs were examined for the presence of clinical signs characteristic of  
88 leptospirosis. For leptospire isolation, blood and urine samples were collected at regular  
89 intervals, and a kidney and liver (study 4) sample were aseptically taken at necropsy. Blood  
90 was also sampled for serological, haematological, and biochemical analysis (study 4 only). At  
91 the end of the observation period or at death, dogs were necropsied, and organs were removed  
92 for histological examination

93

### 94 2.2 Vaccines

95

96 Routine production batches of EURICAN L (Merial, Lyon, France), a whole cell, non-  
97 adjuvanted vaccine prepared from inactivated cultures of *L. interrogans* serovars  
98 icterohaemorrhagiae and canicola, were used. All batches complied with the potency  
99 requirements of monograph 0447 of the European Pharmacopoeia (2002). In studies 1 and 2,  
100 the vaccine was administered simultaneously, but at a separate site, with a vaccine containing  
101 a recombinant canine distemper virus and modified live canine adenovirus type 2, canine  
102 parvovirus, canine coronavirus, and canine parainfluenza type 2 virus. In studies 3 and 4,  
103 EURICAN L was used as diluent to reconstitute a freeze-dried pellet containing a modified

104 live canine distemper virus, canine adenovirus type 2, canine parvovirus and canine  
105 parainfluenza type 2 virus. This second combination vaccine is commercialized under the  
106 name EURICAN DHPPi2L.

107

### 108 **2.3 Animals**

109 Seventy-four specific pathogen free (SPF) 8- to 16-week-old male and female beagle pups  
110 were purchased from Harlan Sprague Dawley (Indianapolis, USA or Zeist, The Netherlands)  
111 or from Ferme des Gouttes (Charles Rivers Laboratories, Inc., France). Dogs were barrier  
112 maintained and fed a high-quality commercial dry ration with unlimited access to water. Dogs  
113 were identified by a microchip implanted subcutaneously and/or by ear tattoo.

114

### 115 **2.4 Challenge strains**

116

117 *L. interrogans* serovar canicola, strain Moulton (National Veterinary Services Laboratory  
118 (NVSL), Ames, Iowa, USA) was used as challenge inoculum in studies 1 and 3. *L.*  
119 *interrogans* serovar icterohaemorrhagiae, strain CFI (NVSL) and strain 193 (Pasteur Institute,  
120 Paris, France) were used as challenge inocula in studies 2 and 4, respectively. The identity of  
121 all serovars was confirmed by the Pasteur Institute, Paris, France, using restriction fragment  
122 analysis.

123

### 124 **2.5 Challenge protocol**

125

126 After an initial culture in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium, the  
127 strains were back-passaged twice (studies 3 and 4) or four times (studies 1 and 2) in hamsters  
128 to prevent loss of virulence through adaptation to culture conditions. Moribund hamsters were  
129 humanely euthanised and their livers and kidneys or spleens (study 4) were aseptically  
130 removed and homogenated in sterile saline. After sedimentation by centrifugation, the  
131 supernatant was diluted 1:10 in sterile saline and inoculated in the dogs of studies 1 and 2.  
132 Each dog received 8 mL of challenge suspension containing approximately  $5 \times 10^8$  and  $1 \times 10^9$   
133 organisms of *L. interrogans* serovars icterohaemorrhagiae and canicola, respectively by the  
134 intraperitoneal route. In studies 3 and 4, after harvest, the challenge strains were passaged  
135 once in vitro (EMJH medium) to allow a more precise quantification of the bacterial  
136 suspension. Each dog received 11 mL (study 3) or 12 mL of challenge suspension with 0.5  
137 mL instilled in the ventral conjunctival sac of each eye and the remainder administered intra-

138 peritoneally. The total challenge dose per dog was  $2.1 \times 10^9$  and  $5.6 \times 10^9$  organisms for *L.*  
139 *interrogans* serovars icterohaemorrhagiae and canicola, respectively.

140

## 141 **2.6 Clinical examination**

142

143 All animals were observed daily for 14 (studies 1 and 2) or 35 days (studies 3 and 4) after  
144 challenge for signs consistent with leptospirosis, including, depression, anorexia,  
145 conjunctivitis, iritis, vomiting, diarrhoea, jaundice, petechiae, and signs of urinary disease  
146 (haematuria). Signs were scored by use of a standardized protocol (Table 2). Rectal  
147 temperatures were taken and recorded daily for 14 days after challenge, and temperatures of  
148  $39.5^\circ\text{C}$  or more were considered as hyperthermia. Dogs from studies 3 and 4 were weighed  
149 once a week until the end of the study or death. A weight loss of more than 5% was  
150 considered significant. During the post-challenge clinical examination, any animals displaying  
151 serious and irreversible clinical signs that lead to suffering were humanely euthanized.

152

## 153 **2.7 Serology**

154

155 Whole blood was collected at regular intervals before and after vaccination and challenge.  
156 Selected sera were tested for the presence of microscopic agglutination titres (MAT) by the  
157 College of Veterinary Medicine, Diagnostic Laboratory, University of Minnesota, St Paul,  
158 USA (studies 1 and 2) or AFFSA, Laboratoire de Recherche Vétérinaire, Alfort, Paris, France  
159 (study 4). Sera were tested against *L. interrogans* serovars icterohaemorrhagiae and canicola  
160 using standardized procedures. Since serology has limited value for evaluating the efficacy of  
161 vaccines against leptospirosis, sera from study 3 were not tested. Antibody titers were  
162 expressed as the reciprocal of the highest serum dilution that induced at least 50% (studies 1  
163 and 2) or 75% (study 4) agglutination. For the calculation of geometric mean titer (GMT),  
164 values under the lower limit of quantification (LLOQ) were replaced by  $\text{LLOQ}/2$ .

165

## 166 **2.8 Haematology**

167

168 EDTA blood samples were collected from dogs of studies 3 and 4 on at least 2 days before  
169 challenge and then daily for 7 days after challenge. Counts of platelets were performed using  
170 a MS-9 cell counter analyser (Melet Schloesing, France). Platelet counts were compared to  
171 reported standard values for dogs ( $2\text{--}9 \times 10^{11}$  - platelets/L (Merck Veterinary Manual, 2005)).

## 172 **2.9 Blood biochemistry**

173  
174 The following tests were only performed on dogs from study 4. Whole blood samples were  
175 collected before challenge and on day 4 and 6 after challenge. Sera were analyzed for urea  
176 nitrogen, creatinine, total bilirubin, serum glutamic oxalacetic transaminase (SGOT), and  
177 serum glutamine pyruvic transaminase (SGPT) by the Laboratoire Marcel Mérieux, Lyon,  
178 France. Urea nitrogen, creatinine, and total bilirubin were compared to normal values  
179 provided by the same laboratory. Because many pre-challenge values for SGOT and SGPT  
180 were outside the reported “normal” values, only large modifications of the baseline values  
181 were taken into account.

182

## 183 **2.10 Detection of leptospiraemia**

184  
185 Blood samples were collected on heparin tubes before challenge (day -2/day 0) and on day 1,  
186 2, 3, 4, 5, 6, 7, and 10 after challenge. In study 4, an additional blood sample was taken on day  
187 35. Blood samples were immediately inoculated in semisolid medium (1-3 drops of blood in 8  
188 mL of medium (studies 1 and 2)) or in liquid EMJH medium (1 mL of blood in 9 mL medium  
189 (studies 3 and 4)) and transferred to the lab. Serial 10-fold dilutions (up to  $10^{-3}$ ) were made in  
190 the same media and incubated at 30°C. All the cultures were incubated for 6-9 weeks and  
191 observed weekly for the presence of leptospire using dark field microscopy.

192

## 193 **2.11 Detection of leptospire in urine and organs**

194  
195 Urine samples were collected before challenge (day -2/day 0) and at 2, 3 and 5 weeks after  
196 challenge (studies 3 and 4) or by direct bladder tap at the time of euthanasia (studies 1 and 2).  
197 In studies 3 and 4, urine samples were collected either spontaneously after subcutaneous  
198 injection of the diuretic furosemide (DIMAZON<sup>®</sup>, Intervet, France) (0.5 to 1 mL/kg  
199 bodyweight) (females) or after probing with a urethra catheter (males). 5 Fluorouracil was  
200 added at a concentration of 100 µg/mL to the urine samples of study 4.

201 Samples from kidneys (all studies) and livers (study 4) were collected aseptically.  
202 Approximately 5-8 grams of organ tissue were macerated into 10 mL of culture medium and  
203 vortexed. Tissue debris was allowed to settle, and serial 10-fold dilutions were made through  
204 1:1,000. Urine and organ cultures were made as described for the blood cultures.

205



## 206 **2.12 Post-mortem examination**

207  
208 Immediately after euthanasia or death, the animals were necropsied and subjected to a  
209 macroscopic examination. Samples of kidneys and livers were fixed with 10% buffered  
210 formalin or frozen and processed for microscopic examination following standard procedures.  
211 Only organ samples from study 4 were submitted for microscopic examination. Histological  
212 sections were stained with haematoxylin-eosin (HE) and with Warthin-Starry silver stain for  
213 the detection of leptospires.

214

## 215 **2.13 Analysis of the results**

216

217 Statistical analyses were carried out using STATGRAPHICS<sup>®</sup> software and SAS<sup>®</sup> release 12  
218 software. The level of significance was set at  $P \leq 0.05$ .

219

## 220 **Clinical scores**

221

222 The severity of clinical signs (sickness score) was compared among the vaccinated and  
223 control groups within one study by assigning the dogs to one of two disease categories: no or  
224 mild clinical disease and moderate-to-severe clinical disease. The sickness score was  
225 calculated by using the daily scores for each clinical sign on the basis of an algorithm, which  
226 gave a triple weighting to the scores for jaundice and haematuria. Thus, sickness score = 1x  
227 (daily score for conjunctivitis/iritis) + 1x (daily score for anorexia) + 1x (daily score for  
228 diarrhoea/vomiting) + 1x (daily score for general appearance) + 3x (daily score for jaundice)  
229 + 3x (daily score for haematuria). Each dog was classified according to the most severe daily  
230 score recorded during the after challenge observation period with a score of 0 for no disease, 1  
231 to 2 for mild disease, 3 to 4 for moderate disease, and >4 for severe disease. Differences in the  
232 incidence of moderate-to-severe disease (scores  $\geq 3$ ) among groups were analyzed by use of a  
233 Fischer's exact test.

234

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237 *registered trademark of SAS Institute Inc. in the United States of America and elsewhere*

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240

### 241 **Leptospiremia**

242

243 Because no leptospiremia was found in the vaccinated pups from studies 1 & 2, no statistical  
244 analysis was performed.

245 A daily score between 0 and 3 was attributed to each animal from studies 3 and 4, according  
246 to the result of the blood culture (0=negative, 1=positive at dilution  $10^{-1}$ , 2=positive at dilution  
247  $10^{-2}$ , 3=positive at dilution  $10^{-3}$ ). The duration of leptospiraemia and cumulative scores for the  
248 first 7 days post challenge (Area under the time-titer curve) were compared between  
249 vaccinated and control dogs using a one-sided student t-test (study 3) or Wilcoxon test (study  
250 4).

251

### 252 **Renal carrier state**

253

254 Any dog with at least one positive urine or kidney culture was defined as a renal carrier.  
255 Differences in the incidence of renal carriers among groups were analyzed by use of a  
256 Fischer's exact test.

257

### 258 **Platelet counts**

259

260 Platelet counts of vaccinated and adult control groups were compared using a Wilcoxon test  
261 (study 4) or a mixed model with repeated measurements (study 3). SGOT and SGPT values of  
262 vaccinated and control groups were compared using a Wilcoxon test and Chi-square test,  
263 respectively.

264

## 265 **3. Results**

266

### 267 **3.1. Humoral responses to vaccination and challenge**

268

269 Prior to vaccination, none of the dogs had detectable antibody titres against *L. interrogans*  
270 serovars icterohaemorrhagiae or canicola. All vaccinated dogs from studies 1 and 2 had  
271 detectable antibody titres on the day of challenge against *L. interrogans* serovar canicola  
272 (GMT=549, range: 80-1280) and *L. interrogans* serovar icterohaemorrhagiae (GMT=47,  
273 range 20-80). A booster effect was observed in one out of nine and eight out of nine dogs after  
274 *Leptospira interrogans* serovars icterohaemorrhagiae and canicola challenge, respectively.

275 High antibody titres were observed in the surviving controls after *L. interrogans* serovar  
276 icterohaemorrhagiae (GMT=1280) and *L. interrogans* serovar canicola challenge  
277 (GMT=3044, range 1280-10240). After both challenges, antibody titres were higher on  
278 average in surviving controls than in vaccinates.

279 Four out of nine dogs from study 4 had detectable antibody titres against *L. interrogans*  
280 serovar icterohaemorrhagiae 4 weeks after the second vaccination (range: 100-200).  
281 Antibodies persisted until challenge in only one dog. A booster response was observed in all  
282 vaccinates after *L. interrogans* serovar icterohaemorrhagiae challenge. In the same study,  
283 seven out of nine dogs had detectable antibody titres against *L. interrogans* serovar canicola 4  
284 weeks after the second vaccination (range: 200-400), and two out of nine animals still had low  
285 MAT antibody titres 5 days before challenge.

286

### 287 **3.2. Clinical signs**

288

289 The incidences of moderate to severe disease in vaccinated and control dogs from studies 1-4  
290 are shown in Table 3.

291 All eight control pups from study 1 became ill after *L. interrogans* serovar canicola challenge;  
292 seven pups developed severe and one pup moderate disease. The affected pups were  
293 depressed and were frequently observed curled up in their food bowls. Some of these animals  
294 were vomiting, slightly dehydrated, and had haematuria. They also had foul smelling bloody  
295 diarrhoea. One pup developed jaundice on day 4 post-challenge. Four pups with severe  
296 clinical disease were humanely euthanised between day post challenge (DPC) 5 and 6. In  
297 contrast, vaccinated pups showed no or only mild transient signs. Two vaccinated pups had  
298 slight conjunctivitis and one pup developed mild digestive signs lasting for one day. The  
299 incidence of moderate to severe disease was significantly lower in the vaccinated pups than in  
300 the control pups ( $P=0.00004$ , Fisher's exact test)

301 All 10 control pups from study 2 developed clinical signs following *L. interrogans* serovar  
302 icterohaemorrhagiae challenge. Six control pups were humanely euthanised because of severe  
303 disease between DPC 4 and 7. Clinical signs were similar between dogs challenged with *L.*  
304 *interrogans* serovars icterohaemorrhagiae and canicola and included depression, anorexia,  
305 haemorrhagic diarrhoea, vomiting, icterus, and haematuria. Three control pups showed only  
306 mild clinical signs consisting of depression and mild diarrhoea, and one control pup showed  
307 no clinical signs. Only one vaccinated pup was depressed on DPC 2 and 8. The incidence of

308 moderate to severe disease was significantly lower in the vaccinated pups than in the control  
309 pups ( $P=0.0077$ , Fisher's exact test)

310 The two control puppies added at the time of challenge in study 3 died from severe  
311 leptospirosis on DPC 4 and 5, validating the challenge. As expected, clinical signs in the adult  
312 controls were less severe than in the control pups. Nevertheless, four control dogs developed  
313 moderate and one dog severe leptospirosis. The latter dog was humanely euthanised on DPC 7  
314 after having shown characteristic signs of frank leptospirosis. Clinical signs in the moderately  
315 ill dogs included conjunctivitis, mild diarrhoea, dehydration, and weight loss. Five out of  
316 seven vaccinated dogs did not show any clinical signs during the observation period. One  
317 vaccinated dog showed conjunctivitis on DPC 18, and two dogs had mild diarrhoea for 2  
318 consecutive days starting on DPC 23. The incidence of moderate to severe disease was  
319 significantly different between the vaccinated and control dogs ( $P=0.0186$ , Fisher's exact  
320 test).

321 In study 4, one of the two control puppies added at the time of challenge died on DPC 6 and  
322 the other developed severe disease (sickness score of 6) but recovered. Unexpectedly, the  
323 challenge appeared to be very severe for the adult controls. Three out of 10 control dogs had  
324 to be humanely euthanised because of depression, diarrhoea, dehydration, and jaundice (one  
325 dog) on DPC 7 (two dogs) and 23, respectively. Five controls had mild disease consisting of  
326 conjunctivitis, depression, and anorexia. Two controls had no disease. Clinical signs in the  
327 vaccinated dogs were mild (conjunctivitis in one dog) or absent. The incidence of moderate to  
328 severe disease was not significantly different between the vaccinated and control dogs  
329 ( $P=0.124$ , Fisher's exact test). Due to the small number of dogs, the *a posteriori* power of the  
330 test was too low (0.15) to detect a significant difference. Twenty-one dogs per group would be  
331 needed to detect the same difference (30%) with a probability equal to 80%.

332

### 333 3.3. Haematology

334

335 No thrombocytopenia was recorded after challenge in the vaccinated dogs from studies 3 and  
336 4, except for one vaccinated dog on day 1 post *L. interrogans* serovar icterohaemorrhagiae  
337 challenge. In contrast, 40% (study 4) to 75% (study 3) of the controls became  
338 thrombocytopenic after challenge. Over the 1 to 7 days post-challenge period, the platelet  
339 count was significantly lower in the controls than in the vaccinates after *L. interrogans* serovar  
340 canicola challenge ( $P=0.0001$ , Mixed model), and the difference was close to significance  
341 ( $P=0.08$ , Wilcoxon's test) after *L. interrogans* serovar icterohaemorrhagiae challenge.

### 342 **3.4. Biochemistry**

343  
344 Sharp increases in urea, creatinine, bilirubin, SGOT, or SGPT values were found after  
345 challenge in three adult controls from study 4 and in the two control puppies that were added  
346 to study 4 at the time of challenge. All these dogs developed severe clinical disease and all  
347 three adult dogs and one of the two puppies succumbed to the challenge. In contrast, none of  
348 the vaccinates had increased urea, creatinine, or bilirubin values. Large modifications of  
349 baseline values of SGPT were recorded in two vaccinated dogs.

350

### 351 **3.5. Leptospiraemia**

352  
353 An overview of the results is provided in Figures 1A (study 1) and 1B (study 2) and Tables  
354 4A (Study 3) and 4B (study 4). All blood samples from studies 1-4 were negative for  
355 leptospires before challenge. Leptospires could be isolated from the blood of all controls from  
356 studies 1 and 2 for at least 3 days following challenge. Leptospiraemia persisted for up to 6  
357 and 10 days for *Leptospira interrogans* serovars icterohaemorrhagiae and canicola,  
358 respectively. None of the vaccinated dogs from studies 1 and 2 developed leptospiraemia  
359 indicating that infection was not established in any of the vaccinated dogs.

360 All control puppies from studies 3 and 4 added at the time of challenge developed  
361 leptospiraemia. Leptospires could be isolated from all vaccinated and control dogs of study 3.  
362 The total amount of leptospira isolated from the blood over the first 7 days after challenge was  
363 significantly lower in the vaccinated dogs than in the control dogs ( $P < 0.0001$ , one-sided  
364 student t-test). In addition, the duration of leptospiraemia was significantly shorter in the  
365 vaccinated dogs compared to the control dogs ( $P = 0.0002$ , student t-test).

366 All control dogs and seven out of nine vaccinated dogs from study 4 developed  
367 leptospiraemia. Both amount and duration of leptospiremia were significantly reduced in the  
368 vaccinated dogs compared to the control dogs ( $P = 0.0001$  for both, Wilcoxon's test).

369

### 370 **3.6. Isolation of leptospires from urine, kidney and livers**

371  
372 An overview of the results is given in Figures 1A (study 1) and 1B (study 2) and Tables 4A  
373 (study 3) and 4B (study 4). All urine samples from studies 1-4 were negative for leptospires  
374 before challenge. Leptospires could be isolated from the kidneys of all control dogs and from  
375 the urine of 37.5% and 30% of the control dogs in studies 1 and 2, respectively. In contrast,

376 none of the vaccinated dogs from studies 1 and 2 had any positive urine or kidney cultures at  
377 the time of euthanasia. The proportion of dogs with renal infection, characterized by the  
378 presence of leptospire in urine and/or kidneys, was significantly lower in the vaccinated dogs  
379 compared to the controls dogs in both studies (Table 5)

380 Seven out of 8 adult control dogs from study 3 shed *L. interrogans* serovar canicola in the  
381 urine, and the kidneys of three control dogs were cultured positive. Leptospire could be  
382 isolated from the urine of two vaccinated dogs and from the kidney of one vaccinated dog.  
383 The incidence of renal carriers was significantly lower in the vaccinated dogs than in the  
384 control dogs ( $P=0.035$ , Fisher's exact test, Table 5).

385 Urine could be collected from only nine adult control dogs of study 4. *L. interrogans* serovar  
386 icterohaemorrhagiae could be recovered from the urine of eight dogs and from the kidneys of  
387 five dogs, but not from the livers. None of the vaccinated dogs shed leptospire in the urine,  
388 and leptospire could not be isolated from any of the kidneys or livers at post-mortem  
389 examination. The incidence of renal carriage was significantly lower in the vaccinated dogs  
390 compared to the control dogs ( $P=0.0006$ , Fisher's exact test, Table 5).

391

### 392 **3.7. Necropsy and histopathology**

393

#### 394 **3.7.1. Necropsy**

395

396 The macroscopic lesions detected during necropsy were similar for all dogs from studies 1-4  
397 that died or were humanely euthanised due to terminal illness. Gross findings included  
398 haemorrhages on the surface of the lungs and the abdominal cavity and the presence of red  
399 stained fluid in the pleural and abdominal cavity. The kidneys were enlarged and friable.  
400 When urine was present from these animals, it was tinged with blood up to severely  
401 haematuric. Typically, faecal material was liquid and tinged with blood having a fetid odour.  
402 In some dogs, the sclera, gingival, and subcutaneous tissues were jaundiced. Apart from some  
403 reactive mesenteric lymph nodes, control dogs that survived the experimental infection  
404 appeared normal on gross visual examination, as well as did all vaccinated dogs.

405

#### 406 **3.7.2. Microscopic examination**

407

408 Prominent lesions in the kidneys of terminally ill control dogs included subacute to severe  
409 interstitial glomerulo-nephritis and tubular degeneration. Moderate to severe diffuse hepatic

410 lesions were found in dogs with jaundice, mostly consisting of an acute degenerative hepatitis  
411 characterized by hepato-cellular dissociation and necrosis. Interestingly, in three surviving  
412 control dogs of study 4, there was evidence of sub-acute multi-focal interstitial nephritis  
413 compatible with leptospirosis infection. No specific lesions were found in the vaccinated  
414 dogs. Only the kidneys from dogs diagnosed with acute renal failure stained positive by  
415 Warthin-Starry silver indicating the presence of leptospire.

416

#### 417 **4. Discussion**

418

419 A number of factors must be considered in the design and evaluation of efficacy trials for  
420 canine leptospirosis vaccines. These factors include the age of the dogs, recommended  
421 vaccination schedule, selection of challenge strain, and challenge method. The ultimate goal  
422 of vaccination against leptospirosis is to protect dogs against clinical disease, as well as  
423 against the establishment of a renal carrier state. The latter protection is especially important  
424 because carrier dogs can be a public health hazard when in close contact to humans (Center  
425 for Disease Control, 1972, Trevejo et al, 1998). Therefore, leptospirosis vaccines should be  
426 tested in models that reliably produce the series of clinical signs and renal colonization pattern  
427 that the vaccine is designed to prevent or reduce. Canine leptospirosis has been a difficult  
428 disease to reproduce under experimental conditions and usually requires the use of young  
429 puppies and a high challenge dose (Keenan et al, 1978). Furthermore, clinical signs may vary  
430 depending on the isolate (Greenlee et al, 2004), altered expression of bacterial proteins  
431 resulting from culture passage (Greenlee et al, 2004), and the timing of harvest after hamster  
432 passage (Minke, personal observation). Even when taking these factors into account, reported  
433 infection in control dogs often results in no evident (Klaasen et al, 2003) or only subclinical  
434 disease (Broughton and Scarnell, 1985; Andre-Fontaine et al, 2003; Schreiber et al, 2005b).  
435 In only a few studies has severe lethal disease been reported following experimental infection  
436 of dogs with *L. interrogans* serovar canicola (Schreiber et al, 2005a; Kerr and Marshall, 1974)  
437 or *L. interrogans* serovar icterohaemorrhagiae (Kerr and Marshall, 1974). In our studies,  
438 puppies experimentally infected with *Leptospira interrogans* serovars icterohaemorrhagiae  
439 and canicola developed a spectrum of disease that ranged from mild to lethal in severity.  
440 Renal, hepatic and haematological signs dominated the clinical presentation and supported the  
441 polysystemic nature of leptospira infection. The overall mortality rate in control puppies was  
442 60% and 58% for *Leptospira interrogans* serovars icterohaemorrhagiae and canicola,  
443 respectively. Under these extreme challenge conditions, clinical signs in the vaccinated pups

444 were rare, and when observed, mild and transient in nature. Clinical disease in adult dogs was  
445 less severe, but unexpectedly, we were able to induce morbidity and mortality in adult dogs as  
446 well, further demonstrating the severity of our challenge models. These results are in sharp  
447 contrast with those published by Klaasen et al (2003), where no evident clinical symptoms  
448 associated with canine leptospirosis were observed in the adult control dogs. The reason for  
449 this difference is not clear but may be attributable to the choice of challenge strain and/or  
450 challenge dose. Hematological parameters and blood biochemistry were not intended to be a  
451 major criterion to assess the efficacy of the vaccine, but they supported the diagnosis of  
452 leptospirosis. Thrombocytopenia was the main hematological abnormality observed in control  
453 dogs after challenge, while vaccinated dogs were protected against thrombocytopenia. This  
454 hematological disorder is a common finding in canine leptospirosis (Greene, 1998) and has  
455 been reported after experimental challenge (Tronel et al, 1999, André-Fontaine et al, 2003;  
456 Klaasen et al, 2003; Schreiber et al, 2005a, Schreiber et al, 2005b). Blood biochemistry  
457 illustrated the alteration of hepatic and renal functions in control dogs. Significant changes in  
458 urea nitrogen, creatinine, bilirubin, SGOT, and SGPT were observed only in the control dogs  
459 with severe clinical signs. The increased levels of SGPT in two vaccinated dogs did not  
460 correlate with the clinical observations. It cannot be ruled out that the massive challenge  
461 induced transient liver damage in those dogs. At necropsy, macroscopic examination of the  
462 animals was consistent with clinical signs, and typical lesions of leptospirosis were observed  
463 in dogs succumbing to the challenge. In addition, microscopic analysis showed that even  
464 surviving controls had lesions of interstitial nephritis compatible with leptospirosis, whereas  
465 no specific lesions were observed in the vaccinated dogs.

466 The second objective of our studies was to determine whether EURICAN L would protect  
467 dogs against the development of a renal carrier state. As in many instances, isolation results  
468 on kidney and urine samples were not concordant in our studies, we defined a renal carrier as  
469 a dog with at least one positive urine or kidney culture. Discrepancies between urine and  
470 kidney isolation results have also been reported in the literature and were attributed to the  
471 presence of specific inhibiting enzymes from kidney cells (Faine, 1998), high urine osmolarity  
472 and pH (Nervig and Garrett, 1979), and the fact that leptospire are shed intermittently  
473 (Nervig and Garrett, 1979). Overall we found that 100% of the control pups and 83% of the  
474 adult controls became renal carriers. Despite the heavy challenges, none of the 18 vaccinated  
475 puppies and only two out of the 16 vaccinated adult dogs developed a renal carrier state. It  
476 should be stressed that the challenge doses that we used were probably much higher than  
477 those observed in a natural infection, suggesting that the protection against renal carriage



478 might be almost complete in the field. The literature has conflicting reports on the efficacy of  
479 leptospiral bacterins to protect against the renal carrier state. Much of the variability is likely  
480 the result of differences in the immunogenicity of the bacterins used, as was demonstrated by  
481 Andre Fontaine et al (2003). In that study, only one of the three commercial vaccines  
482 completely protected dogs against the establishment of a renal carrier state shortly after  
483 primo-vaccination in a challenge model that induced no mortality and severe clinical disease  
484 in only one out of the six control puppies. Culture appeared to more sensitive in our hands  
485 than silver staining to detect leptospires in the kidney, with the additional advantage that  
486 infectious material is detected, rather than fragments of the bacteria. We did not explore  
487 alternative detection methods like PCR or immuno-fluorescence.

488 Typical serological findings in the present studies were the relatively low and short-lived  
489 antibody responses against both serovars after vaccination. Several studies have reported low  
490 antibody responses after administration of leptospirosis inactivated vaccines (Andre-Fontaine  
491 et al, 2003; Schreiber et al, 2005b, Klaasen et al, 2003; Steger-Lieb et al, 1999). Furthermore,  
492 no correlation could be established between antibody titers after vaccination and protection  
493 against experimental infection. The absence of correlation has been classically described in  
494 other studies as well (Broughton and Scarnell, 1985; Andre-Fontaine et al, 2003; Klaasen et  
495 al, 2003; Schreiber et al, 2005a,).

496 It is concluded that a primary course of two doses of EURICAN L provided quick onset and  
497 long-term protection against both clinical leptospirosis and the renal carrier stage. This  
498 vaccine should provide veterinarians with a powerful tool to prevent clinical disease in dogs  
499 and zoonotic transmission of leptospirosis to humans.

500

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502

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505

506

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605

606

607 Table 1: Experimental design

| Study | Designation          | Group | # dogs | Challenge     |         |
|-------|----------------------|-------|--------|---------------|---------|
|       |                      |       |        | Time after V2 | Serovar |
| 1     | Onset of immunity    | V     | 9      | 2 weeks       | Lc      |
|       |                      | C     | 8      |               |         |
| 2     | Onset of immunity    | V     | 9      | 2 weeks       | Li      |
|       |                      | C     | 10     |               |         |
| 3     | Duration of immunity | V     | 7      | 14 months     | Lc      |
|       |                      | C     | 8*     |               |         |
| 4     | Duration of immunity | V     | 9      | 14 months     | Li      |
|       |                      | C     | 10*    |               |         |

608 \*2 control pups were added at the time of challenge V=Vaccinated, C=Control, V2 = second

609 vaccination Lc = *L. interrogans* serovar canicola, Li = *L. interrogans* serovar icterohaemorrhagiae

610

611

612 Table 2: Clinical scoring protocol for canine leptospirosis

613

| <b>Clinical sign</b>  | <b>Degree</b> | <b>Score</b> |
|-----------------------|---------------|--------------|
| Conjunctivitis/Iritis | Absent        | 0            |
|                       | Present       | 1            |
| General appearance    | Normal        | 0            |
|                       | Apathy        | 1            |
|                       | Depression    | 2            |
|                       | Prostration   | 3            |
| Diarrhoea/Vomiting    | Absent        | 0            |
|                       | Mild          | 1            |
|                       | Severe        | 2            |
| Anorexia              | Absent        | 0            |
|                       | Present       | 1            |
| Jaundice              | Absent        | 0            |
|                       | Present       | 1            |
| Haematuria            | Absent        | 0            |
|                       | Present       | 1            |

614

615

616 Table 3: Incidence of moderate to severe disease after challenge

| Study | Group | Disease incidence (No. of dogs) |                     | P-value** |
|-------|-------|---------------------------------|---------------------|-----------|
|       |       | No to Mild                      | Moderate to severe* |           |
| 1     | V     | 9                               | 0                   | 0.00004   |
|       | C     | 0                               | 8 (4)               |           |
| 2     | V     | 9                               | 0                   | 0.0077    |
|       | C     | 4                               | 6 (6)               |           |
| 3     | V     | 7                               | 0                   | 0.0186    |
|       | C     | 3                               | 5 (1)               |           |
| 4     | V     | 9                               | 0                   | 0.124     |
|       | C     | 7                               | 3 (3)               |           |

617

618 Abbreviations: V= vaccinated; C= control.

619 \* In brackets the number of dogs that died or had to be euthanized after challenge.

620 \*\* Fisher's exact test

621 Study 1 = onset of immunity *L. interrogans* serovar canicola622 Study 2 = onset of immunity *L. interrogans* serovar icterohaemorrhagiae623 Study 3 = duration of immunity *L. interrogans* serovar canicola624 Study 4 = duration of immunity *L. interrogans* serovar icterohaemorrhagiae

625

626  
 627 Table 4A: Results of blood, urine and kidney cultures after challenge of dogs with *L*  
 628 *interrogans* serovar canicola. Dogs were challenged 14 months after a primary course of two  
 629 doses of vaccine (study 3: duration of immunity)

630

| 631 Group            | Dog No. | Days after challenge |     |     |     |     |     |     |     |     |   |       |     |     |     |        |     |
|----------------------|---------|----------------------|-----|-----|-----|-----|-----|-----|-----|-----|---|-------|-----|-----|-----|--------|-----|
|                      |         | Blood                |     |     |     |     |     |     |     |     |   | Urine |     |     |     | Kidney |     |
|                      |         | 0                    | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 10  | 0 | 16    | 21  | 35  | 35  |        |     |
| 633 Vaccinated       | 1       | -                    | +++ | +++ | +++ | -   | -   | -   | -   | -   | - | -     | -   | -   | -   | -      | -   |
|                      | 2       | -                    | +++ | +++ | +++ | +++ | +   | -   | -   | -   | - | -     | -   | ++  | +   | -      | -   |
|                      | 634 3   | -                    | +++ | +++ | +++ | +   | -   | -   | -   | -   | - | -     | -   | +   | ++  | +++    | +++ |
|                      | 635 4   | -                    | +++ | +++ | +++ | +   | -   | -   | -   | -   | - | -     | c   | -   | c   | -      | -   |
|                      | 5       | -                    | +++ | ++  | +   | -   | -   | -   | -   | -   | - | -     | -   | -   | -   | -      | -   |
|                      | 636 6   | -                    | +++ | ++  | +   | -   | -   | -   | -   | -   | - | -     | -   | -   | -   | -      | -   |
|                      | 637 7   | -                    | +++ | +++ | ++  | +   | -   | -   | -   | -   | - | -     | c   | -   | -   | -      | -   |
| 638                  | 8       | -                    | +++ | +++ | +++ | +++ | -   | -   | -   | -   | - | c     | +++ | +++ | -   | -      |     |
|                      | 9       | -                    | +++ | +++ | +++ | +++ | +++ | +++ | ++  | -   | - | -     | +++ | +++ | -   | -      |     |
| 639 Control (adults) | 10      | -                    | +++ | +++ | +++ | +++ | ++  | -   | ++  | -   | - | c     | +++ | +   | +++ | +++    |     |
|                      | 640 11  | -                    | +++ | +++ | +++ | +++ | +   | -   | +   | -   | - | -     | ++  | +++ | -   | -      |     |
| 641                  | 12      | -                    | +++ | +++ | +++ | +++ | +++ | +++ | ++  | -   | - | -     | +++ | c   | +++ | -      |     |
|                      | 641 13  | -                    | +++ | +++ | +++ | +++ | -   | +   | +   | -   | - | c     | +++ | c   | +++ | +++    |     |
| 642                  | 14      | -                    | +++ | +++ | +++ | +++ | +++ | -   | -   | -   | - | c     | -   | c   | -   | -      |     |
|                      | 642 15  | -                    | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | d | -     | ++  | d   | d   | +++    |     |
| Control (puppies)    | 16      | -                    | +++ | +++ | +++ | d   | d   | d   | d   | d   | d | c     | d   | d   | d   | +++    |     |
|                      | 17      | -                    | +++ | +++ | +++ | +++ | d   | d   | d   | d   | d | -     | d   | d   | d   | c      |     |

+++ culture positive at dilution 1/1000

++ culture positive at dilution 1/100

+ culture positive at dilution 1/10

- culture negative

c=contaminated

d= died or euthanised



643 Table 4B: Results of blood, urine and kidney cultures after challenge of dogs with *L.*  
 644 *interrogans* serovar icterohaemorrhagiae. Dogs were challenged 14 months after a primary  
 645 course of two doses of vaccine (study 4: duration of immunity)

646

| Group             | Dog No. | Days after challenge |     |     |     |     |     |   |   |   |   |   | Urine |     |     |    | Kidney       |    |              |   |   |   |   |   |   |
|-------------------|---------|----------------------|-----|-----|-----|-----|-----|---|---|---|---|---|-------|-----|-----|----|--------------|----|--------------|---|---|---|---|---|---|
|                   |         | Blood                |     |     |     |     |     |   |   |   |   |   | -2    | 14  | 21  | 35 | day of death | 35 | day of death |   |   |   |   |   |   |
| Vaccinated        | 1       | -                    | +++ | -   | -   | -   | -   | - | - | - | - | - | -     | -   | -   | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 2       | -                    | +   | -   | -   | -   | -   | - | - | - | - | - | -     | -   | -   | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 3       | -                    | +   | -   | -   | -   | -   | - | - | - | - | - | -     | -   | -   | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 4       | -                    | -   | -   | -   | -   | -   | - | - | - | - | - | -     | -   | -   | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 5       | -                    | -   | -   | -   | -   | -   | - | - | - | - | - | -     | -   | -   | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 6       | -                    | +   | -   | -   | -   | -   | - | - | - | - | - | -     | -   | -   | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 7       | -                    | ++  | -   | -   | -   | -   | - | - | - | - | - | -     | -   | -   | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 8       | -                    | +   | +   | -   | -   | -   | - | - | - | - | - | -     | -   | -   | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 9       | -                    | ++  | -   | -   | -   | -   | - | - | - | - | - | -     | -   | -   | -  | -            | -  | -            | - | - | - | - | - | - |
| Control (adults)  | 10      | -                    | +++ | +++ | +++ | -   | -   | - | d | d | d | - | d     | d   | d   | NS | d            | -  | -            | - | - | - | - | - |   |
|                   | 11      | -                    | +++ | +++ | +   | -   | +   | - | - | - | - | - | +++   | +++ | -   | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 12      | -                    | +++ | +++ | +   | -   | -   | - | - | - | - | - | +++   | +++ | +++ | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 13      | -                    | +++ | +++ | +   | -   | -   | - | - | - | - | - | +++   | +++ | ++  | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 14      | -                    | +++ | +++ | +   | -   | -   | - | - | - | - | - | +++   | +++ | -   | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 15      | -                    | +++ | +++ | +++ | +++ | +++ | - | - | d | d | - | d     | d   | d   | +  | d            | -  | -            | - | - | - | - | - | - |
|                   | 16      | -                    | +++ | +++ | +   | -   | -   | - | - | - | - | - | +++   | +++ | ++  | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 17      | -                    | +++ | +++ | +++ | -   | -   | - | - | - | - | - | +++   | +++ | -   | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 18      | -                    | +++ | -   | +   | -   | -   | - | - | - | - | - | -     | -   | -   | -  | -            | -  | -            | - | - | - | - | - | - |
|                   | 19      | -                    | +++ | +++ | ++  | -   | -   | - | - | - | - | - | +++   | +++ | d   | +  | d            | -  | -            | - | - | - | - | - | - |
| Control (puppies) | 20      | -                    | +   | +   | +   | +   | +   | + | d | d | d | - | d     | d   | d   | +  | d            | -  | -            | - | - | - | - | - |   |
|                   | 21      | -                    | +   | +   | +   | +   | -   | + | - | - | - | - | +     | +   | +   | -  | +            | -  | -            | - | - | - | - | - |   |

+++ culture positive at dilution 1/1000

++ culture positive at dilution 1/100

+ culture positive at dilution 1/10

- culture negative

c=contaminated

d= died or euthanised

NS = no sample

648 Table 5: Incidence of renal carrier state after challenge (Any dog with at least one positive  
649 urine or kidney culture was defined as a renal carrier)

| Study | Group | Incidence of renal carries (No. of dogs) |         | P-value* |
|-------|-------|--|---------|----------|
|       |       | Absent                                   | Present |          |
| 1     | V     | 9  | 0       | 0.00005  |
|       | C     | 0  | 8       |          |
| 2     | V     | 9  | 0       | 0.00001  |
|       | C     | 0  | 10      |          |
| 3     | V     | 5  | 2       | 0.035    |
|       | C     | 1  | 7       |          |
| 4     | V     | 9  | 0       | 0.0006   |
|       | C     | 2  | 8       |          |

650 Study 1 = Onset of immunity study *L. interrogans* serovar canicola

651 Study 2 = Onset of immunity study *L. interrogans* serovar icterohaemorrhagiae

652 Study 3 = Duration of immunity study *L. interrogans* serovar canicola

653 Study 4 = Duration of immunity study *L. interrogans* serovar icterohaemorrhagiae

654 Abbreviations: V= vaccinated; C=control

655 \*Fisher's exact test.

656

657 Legends to illustrations

658

659

660 Figure 1A : Results of blood, urine and kidney cultures after challenge of puppies with *L.*  
661 *interrogans* serovar canicola. Puppies were challenged two weeks after a primary course of  
662 two doses of vaccine (study 1: onset of immunity)

663

664 Figure 1B : Results of blood, urine and kidney cultures after challenge of puppies with *L.*  
665 *interrogans* serovar icterohaemorrhagiae. Puppies were challenged two weeks after a primary  
666 course of two doses of vaccine (study 2: onset of immunity)

667

668

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Figure 1A :

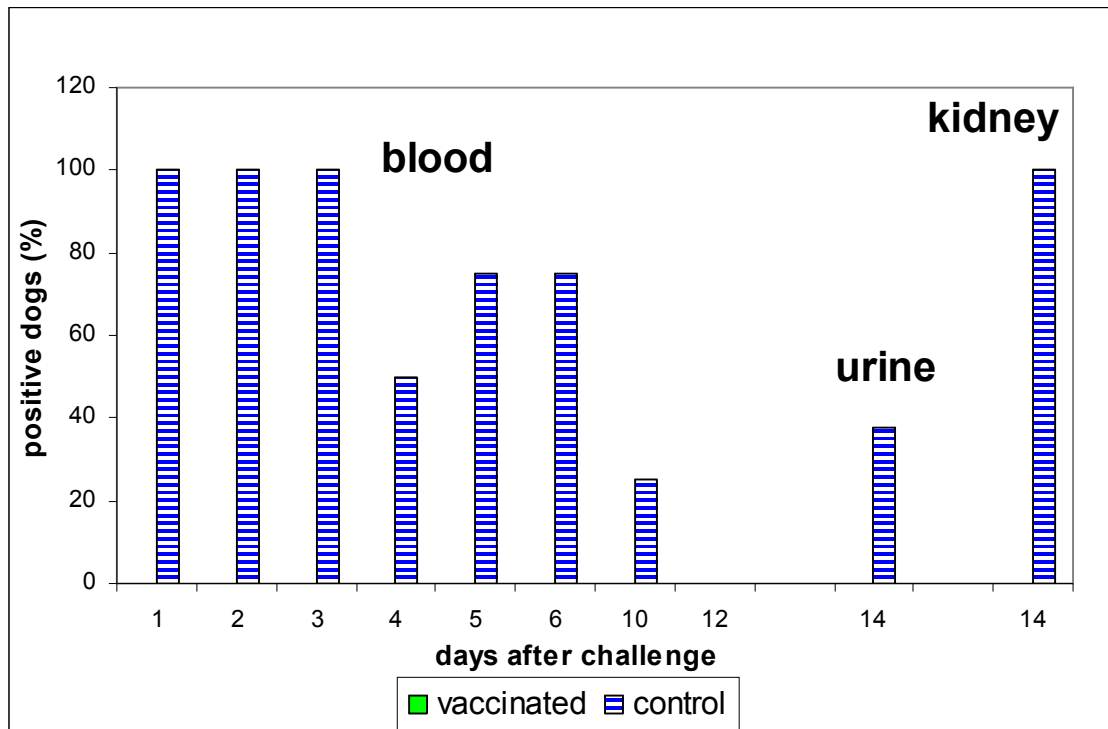
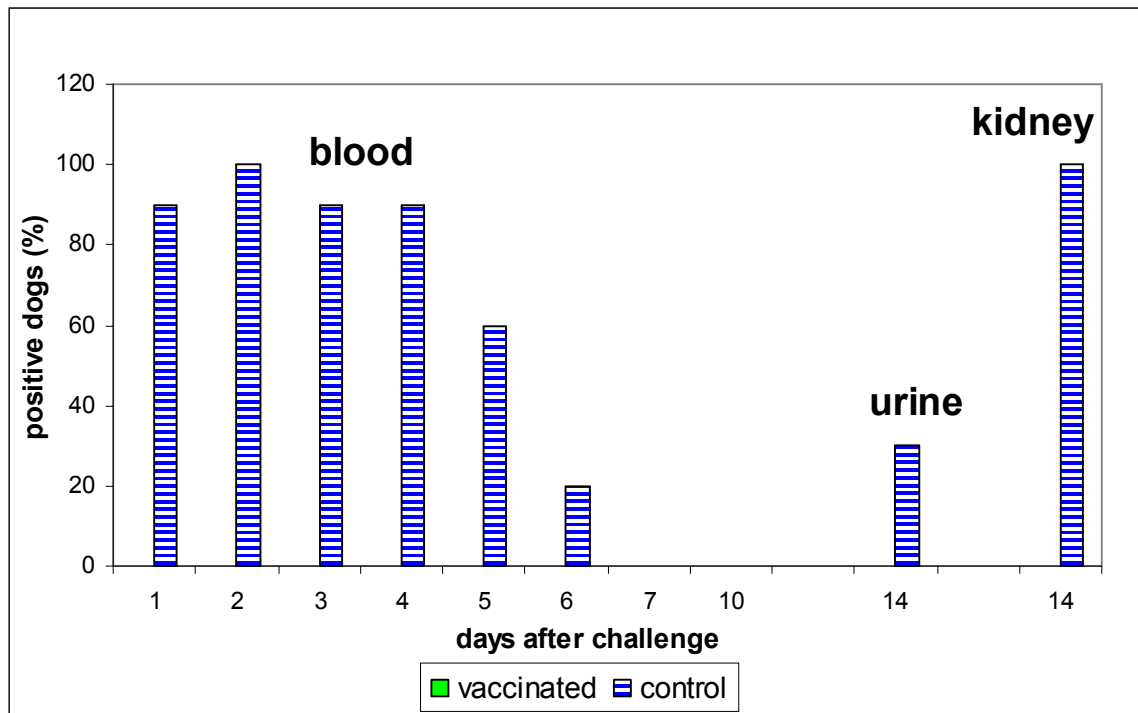


Figure 1B :



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