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11 Abstract

12

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

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

33

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35

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

38

39 **1. Introduction**

40

Leptospirosis is an important zoonosis of worlwide distribution caused by infection with 41 spirochaetes belonging to the pathogenic species of Leptospira. Infection typically results 42 from direct or indirect contact with urine of infected animals. The clinical signs associated 43 with Leptospira infection range from subclinical to acute disease characterized by anorexia, 44 vomiting, lethargy, muscle pain, dehydration, jaundice, abdominal pain, diarrhoea, bloody 45 urine, and death. Renal failure is the predominant finding in symptomatic dogs, with a small 46 percentage also showing evidence of liver disease (Greene, 1998, review; Boutilier and 47 Schulman, 2003). Clinically recovered dogs frequently become asymptomatic renal carriers, 48 and as such can be an important source of human leptospirosis (Center for Disease Control 49 1972, Trevejo et al., 1998). Leptospira (L) interrogans serovars icterohaemorrhagiae and 50 canicola are the two serovars traditionally associated with disease in dogs (Hartman, 1984, 51 52 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, 53 54 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 55 in dogs after vaccination with several vaccines (Huhn et al, 1975; Andre-Fontaine et al, 2003; 56 Klaasen et al, 2003; Schreiber et al, 2005a, Schreiber et al, 2005b), there is much debate on 57 whether leptospirosis vaccines protect against the renal carrier state (Andre Fontaine et al, 58 2003) or provide long term immunity (Cohne et al., 2001). As far as we know, we were the 59 first to demonstrate 10 months duration of immunity against L. interrogans servor canicola 60 provided by a classical bacterin (Tronel et al, 1999). Since then only one paper has been 61 published demonstrating duration of immunity of 13 months for a commercial bacterin 62 (Klaasen et al. 2003). In the current study, we confirm and extend our previous observations 63 and demonstrate that two doses of EURICAN® L provide both rapid onset and duration of 64 protective immunity of at least 14 months against both serovars icterohaemorrhagiae and 65 canicola. The vaccine was evaluated for protection against clinical disease and prevention of 66 the renal carrier state. 67

70 2. Materials and methods

71

72 2.1 Experimental design

73

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

93

94 **2.2 Vaccines**

95

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

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

107

108 **2.3 Animals**

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

114

115 **2.4 Challenge strains**

116

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

123

124 **2.5 Challenge protocol**

125

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

140

141 **2.6 Clinical examination**

142

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

152

153 **2.7 Serology**

154

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

165

166 **2.8 Haematology**

167

EDTA blood samples were collected from dogs of studies 3 and 4 on at least 2 days before challenge and then daily for 7 days after challenge. Counts of platelets were performed using a MS-9 cell counter analyser (Melet Schloesing, France). Platelet counts were compared to reported standard values for dogs $(2-9x10^{11} - platelets/L (Merck Veterinary Manual, 2005))$.

172 **2.9 Blood biochemistry**

173

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

182

183 **2.10 Detection of leptospiraemia**

184

Blood samples were collected on heparin tubes before challenge (day -2/day 0) and on day 1, 2, 3, 4, 5, 6, 7, and 10 after challenge. In study 4, an additional blood sample was taken on day 35. Blood samples were immediately inoculated in semisolid medium (1-3 drops of blood in 8 mL of medium (studies 1 and 2)) or in liquid EMJH medium (1 mL of blood in 9 mL medium (studies 3 and 4)) and transferred to the lab. 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 6-9 weeks and observed weekly for the presence of leptospires using dark field microscopy.

192

193 **2.11 Detection of leptospires in urine and organs**

194

Urine samples were collected before challenge (day -2/day 0) and at 2, 3 and 5 weeks after challenge (studies 3 and 4) or by direct bladder tap at the time of euthanasia (studies 1 and 2). In 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) or after probing with a urethra catheter (males). 5 Fluorouracil was added at a concentration of 100 μ g/mL to the urine samples of study 4.

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

206 2.12 Post-mortem examination

207

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

214

215 **2.13 Analysis of the results**

216

Statistical analyses were carried out using STATGRAPHICS[®] software and SAS[®] release 12 software. The level of significance was set at $P \le 0.05$.

219

220 Clinical scores

221

The severity of clinical signs (sickness score) was compared among the vaccinated and 222 control groups within one study by assigning the dogs to one of two disease categories: no or 223 224 mild clinical disease and moderate-to-severe clinical disease. The sickness score was calculated by using the daily scores for each clinical sign on the basis of an algorithm, which 225 gave a triple weighting to the scores for jaundice and haematuria. Thus, sickness score = 1x226 (daily score for conjunctivitis/iritis) + 1x (daily score for anorexia) + 1x (daily score for 227 diarrhoea/vomiting) + 1x (daily score for general appearance) + 3x (daily score for jaundice) 228 + 3x (daily score for haematuria). Each dog was classified according to the most severe daily 229 score recorded during the after challenge observation period with a score of 0 for no disease, 1 230 to 2 for mild disease, 3 to 4 for moderate disease, and >4 for severe disease. Differences in the 231 incidence of moderate-to-severe disease (scores ≥ 3) among groups were analyzed by use of a 232 Fischer's exact test. 233

234

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- 237 registered trademark of SAS Institute Inc. in the United States of America and elsewhere
- 238
- 239
- 240

241 Leptospiremia

242

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

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

251

252 **Renal carrier state**

253

Any dog with at least one positive urine or kidney culture was defined as a renal carrier.

Differences in the incidence of renal carriers among groups were analyzed by use of a
Fischer's exact test.

257

258 Platelet counts

259

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

264

3. Results

266

3.1. Humoral responses to vaccination and challenge

268

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

High antibody titres were observed in the surviving controls after *L. interrogans* serovar interrogans (GMT=1280) and *L. interrogans* serovar canicola challenge

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

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

286

275

287 **3.2. Clinical signs**

288

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

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

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

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

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

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

332

333 **3.3. Haematology**

334

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

342 **3.4. Biochemistry**

343

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

350

351 **3.5. Leptospiraemia**

352

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

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

All control dogs and seven out of nine vaccinated dogs from study 4 developed leptospiraemia. Both amount and duration of leptospiremia were significantly reduced in the 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

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

before challenge. Leptospires could be isolated from the kidneys of all control dogs and from

the urine of 37.5% and 30% of the control dogs in studies 1 and 2, respectively. In contrast,

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

Seven out of 8 adult control dogs from study 3 shed *L. interrogans* serovar canicola in the urine, and the kidneys of three control dogs were cultured positive. Leptospires could be isolated from the urine of two vaccinated dogs and from the kidney of one vaccinated dog. The incidence of renal carriers was significantly lower in the vaccinated dogs than in the 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 leptospires in the urine, ³⁸⁸ and leptospires 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

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

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

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

416

417 **4. Discussion**

418

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

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

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

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

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

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

500

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502

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607 Table 1: Experimental design

Study	Designation	Group	# dogs	Challe	nge	
				Time after V2	Serovar	
1	Onset of immunity	V	9	2 weeks	Ic	
1	Onset of minimunity	С	8			
2	Onset of immunity	V	9	2 weeks	Li	
	Onset of minimunity	С	10			
3	Duration of immunity	V	7	14 months	Lc	
5	Duration of minianity	С	8*			
4	Duration of immunity	V	9	14 months	Li	
- T	Duration of minimulity	С	10*		LI	

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

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

- 610
- 611

612 <u>Table 2</u>: Clinical scoring protocol for canine leptospirosis

613

Clinical sign	Degree	Score			
Conjunctivitie/Iritie	Absent	0			
Conjunctivitis/infus	Present	1			
	Normal	0			
Conoral appearance	Apathy	1			
General appearance	Depression	2			
	Prostration	3			
	Absent	0			
Diarrhoea/Vomiting	Mild	5			
	Severe	2			
Anorovia	Absent	0			
Anorexia	Present	1			
Joundiaa	Absent	0			
Jaunaice	Present	1			
Haamaturia	Absent	0			
Haematuna	Present	1			

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Study.	Crown	Disease incid	lence (No. of dogs)	D voluo**
Study	Group	No to Mild	Moderate to severe*	- r-value""
1	V	9	0	0.00004
1	С	0	8 (4)	0.00004
2	V	9	0	0.0077
2	С	4	6 (6)	- 0.0077
3	V	7	0	0.0186
2	С	3	5 (1)	0.0100
1	V	9	0	0.124
7	С	7	3 (3)	0.124

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

617

Abbreviations: V= vaccinated; C= control. 618

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

****** Fisher's exact test 620

Study 1 = onset of immunity *L. interrogans* serovar canicola 621

Study 2 = onset of immunity *L. interrogans* serovar icterohaemorrhagiae 622

Study 3 = duration of immunity *L. interrogans* serovar canicola 623

Study 4 = duration of immunity *L. interrogans* serovar icterohaemorrhagiae 624 ııty

626

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

630

631 Group	Dog No.	Days	after cl	halleng	ge										
632	-	Blood	l								Urine				Kidney
052		0	1	2	3	4	5	6	7	10	0	16	21	35	35
633	1	-	+++	+++	+++	-	-	-	-	-	-	-		-	-
(a)	2	-	+++	+++	+++	+++	+	-	-	-	-	++	+	-	-
634	3	-	+++	+++	+++	+	-	-	-	-	-	+	++	+++	+++
63 Vaccinated	4	-	+++	+++	+++	+	-	-	-	-	c	-	c	-	-
05 waccinated	5	-	+++	++	+	-	-	-	-	-	-	_	_)	-	-
636	6	-	+++	++	+	-	-	-	-	-	-	<u> </u>	-	-	-
637	7	-	+++	+++	++	+	-	-	-	-	с	-	-	-	-
638	8	-	+++	+++	+++	+++	-	-	-	-	С	+++	+++	-	-
038	9	-	+++	+++	+++	+++	+++	+++	++	-	-	+++	+++	-	-
639	10	-	+++	+++	+++	+++	++	-	++	_	с	+++	+	+++	+++
Gantral	11	-	+++	+++	+++	+++	+	-	+	-	-	++	+++	-	-
6400introl	12	-	+++	+++	+++	+++	+++	+++	++	(-)	-	+++	c	+++	-
(aduits) 641	13	-	+++	+++	+++	+++	-	+	+	-	c	+++	c	+++	+++
041	14	-	+++	+++	+++	+++	+++	-	-	-	c	-	c	-	-
642	15	-	+++	+++	+++	+++	+++	+++	+++	d	-	++	d	d	+++
Control	16	-	+++	+++	+++	d	d	d	d	d	с	d	d	d	+++
(puppies)	17	-	+++	+++	+++	+++	d	d	d	d	-	d	d	d	с

+++ 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

2 D CICIE 2

- Table 4B: Results of blood, urine and kidney cultures after challenge of dogs with L. 643
- interrogans serovar icterohaemorraghiae. Dogs were challenged 14 months after a primary 644
- course of two doses of vaccine (study 4: duration of immunity) 645
- 646

61Group	Dog No.	Days	after cl	nalleng	ge													
047		Blood	1									Urir	ne				Kidney	
		-2	1	2	3	4	5	6	7	10	35	-2	14	21	35	day of death	35	day of death
	1	-	+++	-	-	-	-	-	-	-	-	-	-	-	-		<u> </u>	
Vaccinated	2	-	+	-	-	-	-	-	-	-	-	-	-	-	-		-	
	3	-	+	-	-	-	-	-	-	-	-	-	-	-			-	
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	
	6	-	+	-	-	-	-	-	-	-	-	-	-	-	<u> </u>		-	
	7	-	++	-	-	-	-	-	-	-	-	-	-	-	-)		-	
	8	-	+	+	-	-	-	-	-	-	-	-	-	Ċ	-		-	
	9	-	++	-	-	-	-	-	-	-	-	-		-) -		-	
	10	-	+++	+++	+++	-	-	-	d	d	d	-	d	d	d	NS	d	-
	11	-	+++	+++	+	-	+	-	-	-	-	-	+++	+++	-		-	
	12	-	+++	+++	+	-	-	-	-	-	-	-	+++	+++	+++		+++	
	13	-	+++	+++	+	-	-	-	-	-	-		+++	+++	++		+++	
Control	14	-	+++	+++	+	-	-	-	-	-	-	-	+++	+++	-		-	
(adults)	15	-	+++	+++	+++	+++	+++	-	-	d	d	-	d	d	d	+	d	-
(aduns)	16	-	+++	+++	+	-	-	-	-	-	-	U -	+++	+++	++		++	
	17	-	+++	+++	+++	-	-	-	-	-	-	-	+++	+++	-		+++	
	18	-	+++	-	+	-	-	-	-	-		-	-	-	-		-	
	19	-	+++	+++	++	-	-	-	-	-	-	-	+++	+++	d	+	d	+
Control	20	-	+	+	+	+	+	+	d	d	d	-	d	d	d	+	d	+
(puppies)	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

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

Study	Group	Incidence of renal of	P_voluo*			
Study	Group	Absent	Present			
1	V	9	0	0.00005		
1	С	0	8	0.00003		
2	V	9	0	0.00001		
2	С	0	10	- 0.00001		
3	V	5	2	0.035		
5	С	1	7			
4	V	9	0	0,0006		
•	C	2	8			

650

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

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

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

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

Abbreviations: V= vaccinated; C=control 654

*Fisher's exact test. 655

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
- 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
- course of two doses of vaccine (study 2: onset of immunity)

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Figure 1A :



Figure 1B :

