1 **Characterization of *Leptospira* Infection in Suckling and Weaning Rat Pups**

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17 **ABSTRACT**

18

19 Rats are known to be the most important reservoirs of *Leptospira* spp. However, the
20 leptospiral dose and age at which rats become resistant to *Leptospira* infection are not yet
21 well elucidated. Aimed to characterize leptospirosis in rat pups, we found that suckling pups
22 (4-, 7-, and 14-day old) are susceptible to leptospires and resistance starts from the weaning
23 age (23-day old). Susceptibility of rat pups was also affected by the infecting dose of the
24 organisms. Jaundice, decrease in body weight, and neurological symptoms prior to
25 moribundity was evident in infected suckling pups. However, 23-day-old infected pups did
26 not manifest any pathological changes and were able to survive the infection similar to adult
27 rats. Based on these results, we propose the suckling rat pup as a novel animal model of
28 human leptospirosis to investigate pathogenesis, development of host resistance, and the
29 mechanisms involved in rats becoming maintenance hosts for leptospires.

30

31 **Keywords:** leptospirosis, susceptibility, resistance, maintenance host, animal model

32 1. Introduction

33

34 Leptospirosis, caused by pathogenic *Leptospira*, is well known as a worldwide
35 zoonosis, which is endemic in tropical and subtropical areas [1]. Its clinical manifestations
36 make it difficult to differentiate from other tropical infections [1]. In spite of the fact that this
37 disease is spreading from various animals to other animals and to humans, the mechanisms
38 involved in the pathogenesis of *Leptospira* are still largely unknown, making it a major
39 public health problem [1, 2].

40 *Leptospira* are thin, highly motile and helically coiled bacteria that belong to the
41 family *Leptospiraceae* [1]. Pathogenic leptospires invade the susceptible host's body through
42 abrasions or lesions of the skin or through the mucous membranes. These organisms then
43 spread to all of the organs following circulation through the blood stream [3]. The duration of
44 the bacteremic phase is from 4 to 7 days and would be followed by very mild symptoms or
45 severe illness, which may sometimes lead to death [3, 4]. This outcome of infection may be
46 due to direct effects of the pathogen or genetically determined host immune responses [4].

47 Mild leptospirosis is difficult to distinguish from other infections as it has non-
48 specific symptoms such as fever, chills, headache, muscle aches, abdominal pain, and
49 conjunctival suffusion during the acute phase [4]. On the other hand, jaundice, acute renal
50 failure, and bleeding or hemorrhage in the lungs are characteristics of severe illness, known
51 as Weil's disease [1].

52 Hamsters and guinea pigs have been used as susceptible animal models of
53 leptospirosis [2, 5-9]. The use of gerbils [10] and marmoset monkeys [11] in experimental
54 leptospirosis have also been reported. Although mice are considered resistant to *Leptospira*
55 infection, it has been reported that *tlr4* mutant mice (C3H/HeJ) [12, 13] and

cyclophosphamide-treated mice [14] are susceptible to this infection but frequency and severity of nephritis is lower in *Inos* knockout mice [15]. However, these mouse models of infection do not mimic the natural susceptibility among rodents. Four commonly used wild-type mouse strains (i.e., A/J, CBA, BALB/c, and C57BL/6) that are known to be resistant to lethal infection with *Leptospira* have developed specific pathologies as outcomes of sub-lethal infections [16].

In 1917, rats were found to be one of the sources of human *Leptospira* infection [17]. Rats are known to harbor the bacteria in their kidneys without showing any symptoms of leptospirosis [2, 18, 19]. Also, it is a general maintenance host for serovars belonging to serogroups Icterohaemorrhagiae and Ballum [20]. Based on the experiment conducted by Athanazio et al in 2008, clearance of leptospires in all tissues of rats, except in kidneys, was found within 9 days after infection [18]. Moreover, the experimentally infected rats were found to excrete high dose of leptospires in the urine (around 10^7 leptospires/ml) [21]. Urine excretion in the environment was thought to be one of the probable causes of *Leptospira* transmission among rats in nature. Although there was a report by Kemenes (1965) showing rat body weight limits in fatal cases of *L. interrogans* infection [22], it is still unclear at what age rats become resistant to *Leptospira* infection and the mechanisms by which rats become maintenance hosts for leptospires. In order to explore these, we aimed to characterize leptospiral infection in suckling and weaning rat pups.

75

2. Materials and methods

77

2.1. Rat infection

Pregnant Wistar rats at 14th day of gestation (SLC, Hamamatsu, Japan) were provided with food and drink *ad libitum*. The time immediately after the birth of pups was day 0.

81 Four-, 7-, 14-, and 23-day old pups were then subcutaneously infected with 10^8 , 10^6 , 10^4 and
82 10^2 organisms of *L. interrogans* serovar Manilae strain K64 [23, 24]. Low passage (<10X) *in*
83 *vitro* sub-cultures organisms were suspended in PBS and used for infection [23] (See Section
84 3.2 for the number of pups per group). Pups injected with PBS only were used as controls
85 (uninfected). The pups were monitored daily for their body weight and clinical signs of
86 illness such as loss of appetite, decreased or loss of activity, etc. Pups showing continuous
87 weight loss, jaundice, and loss of activity were considered to be moribund, and were
88 euthanized by inhalation of sevoflurane (Sevofrane, Maruishi Pharmaceutical Co., Ltd, Osaka,
89 Japan). Infected and uninfected rat pups were observed until 30 days post bacterial
90 inoculation.

91

92 2.2. Total bilirubin

93 To measure the amount of total bilirubin in sera, the blood was aseptically collected
94 by cardiac puncture of moribund 7-day old pups injected with 10^2 and 10^8 leptospire (lowest
95 and highest doses) as well as uninfected pups. Total bilirubin in serum was measured by
96 using Bilirubin Kit-K (Alfresa Pharma Corporation, Osaka, Japan). This was done by mixing
97 20 ul of each serum sample with Dyphylline, Diazo and Fehling reagents (200 ul per reagent)
98 and color reaction was measured using spectrophotometer (600 nm).

99

100 2.3. Organ culture

101 Kidneys, spleens, livers, lungs, and brains were collected from 4- and 7-day-old
102 infected pups that became moribund after infection. These organs were aseptically removed,
103 placed in disposable 2.5 ml syringe, and crushed into 4 ml sterile Korthof's medium with 5-
104 fluorouracil (5-FU; 100 μ g/ml), and incubated at 30°C [23, 24]. The next day, 500 μ l of the

105 culture supernatant was sub-cultured in fresh Korthof's medium and further incubated. The
106 presence or absence of *Leptospira* in the cultures was observed until 60 days.

108 2.4. Urine

109 Urine was aseptically collected by massaging the dorsal-caudal area of the pups until
110 urine was excreted. Ten µl of urine was observed directly under dark-field microscope, and
111 ≤50 µl of urine were cultured in Korthof's medium and observed for 60 days. *Leptospira*-
112 positive urine was determined by the presence of leptospires in direct microscopy and/or in
113 urine culture.

115 2.5. Quantitative microscopic agglutination test (MAT)

116 Whole blood was collected from the retro-orbital plexus of surviving 14- and 23-day
117 old infected pups during the 2nd and 4th week post infection. Serum was obtained by
118 centrifuging the whole blood at 3,000 rpm for 30 minutes. The serum samples were then heat
119 inactivated for 30 minutes at 56°C, cooled down, and stored at -20°C until MAT was
120 performed.

121 MAT was done using pathogenic *L. interrogans* serovar Manila strains K64 and
122 saprophytic *L. biflexa* strain Patoc I as antigens to detect the presence of anti-*Leptospira*
123 antibodies and their titers in the sera of rat pups. MAT was carried out using the quantitative
124 method specified in the World Health Organization (WHO)-International Leptospirosis
125 Society (ILS) Guidance on Human Leptospirosis [25]. Titers greater than or equal to 1:20
126 were considered significant (MAT-positive) [23].

128 2.6. Histopathology

Moribund 7-day old K64 (10^8 and 10^2) -infected and uninfected pups were sacrificed by inhalation of sevoflurane. Kidneys, spleens, liver, lungs, and brains were collected for histopathology. These organs were fixed in 10% neutral-buffered-formalin (10% NBF), embedded in paraffin, cut in 4 μ m sections, and stained with haematoxylin-eosin (HE). Immunohistochemistry was performed on paraffin-embedded tissue sections using antiserum against *L. interrogans* serovar Manilae strain LT 398, which was generated in rabbits [24].

2.7 Animal ethics

The protocol for rat experiments were reviewed and approved by the Ethics Committee on Animal Experiment, Faculty of Medical Sciences, Kyushu University. The experiments were performed according to the Regulations for Animal Experiments of the said University.

3. Results

3.1. Symptoms of infection and total bilirubin levels in *Leptospira*-infected rat pups

One day prior to becoming moribund, 4- and 7-day-old K64-infected pups had jaundice, which can be clearly seen in the ears, extremities and tail (Fig. 1A). However, jaundice was observed 4 days prior to moribundity in the 14-day-old infected pups. The appearance of jaundice was followed by neurological symptoms such as body tremors, weakness or movement disorder, and seizures. However, these findings were not observed in the control pups.

Fig. 2 shows that the average level of total bilirubin was 0.78, 5.05, and 6.02 mg/dl for the 7-day-old control, 10^2 , and 10^8 leptospire-infected pups, respectively.

3.2. Survival of rat pups

The survival rate per infecting dose within each age group was compared to the control (n = total number of animals per infecting dose) (Fig. 3). All of the 4-day old pups infected with 10^8 (n=5), 10^4 (n=6), and 10^2 (n=6) K64 died within 5, 7, and 9 days post infection, respectively (*p<0.01). However, 2 out of 6 of the 10^6 K64-infected pups survived the infection (**p<0.05) (Fig. 3A). Among the infected 7-day old pups, all of those infected with 10^8 (n=7) and 10^4 (n=8) doses died within 4 and 7 days post infection, respectively (*p<0.01) (Fig. 3B). On the other hand, pups infected with 10^6 (n=8) and 10^2 (n=8) doses had 25% survival (**p<0.05). Survival rates of 28.6%, 87.5%, 42.9%, and 83.3% were found in 14-day old pups infected with 10^8 (n=7), 10^6 (n=8), 10^4 (n=7), and 10^2 (n=6) leptospire, respectively (*p<0.05) (Fig. 3C). All of the 23-day old infected pups were able to survive leptospiral infection regardless of the infecting dose (data not shown).

3.3. Changes in body weight

A 5% decrease in body weight was observed prior to being moribund in 4- and 7-day old infected pups (Figs. 4A and 4B). This decrease was observed to be corresponding with the appearance of jaundice. However, 14-day old infected pups had as much as 15% decrease in body weight prior to moribundity (Fig. 4C). The 10^4 infecting dose in these ages were statistically different from the control. All of the 23-day old infected pups survived the experimental leptospiral infection but were found to have a slightly lower body weight compared to the control (Fig. 4D).

3.4. *Leptospira* in organ and urine cultures

Organ cultures revealed that leptospire were recovered from almost all of the organs of 4- and 7-day old infected pups but not in the control pups (Table 1). The brain cultures of all 4-day old and kidney cultures of 14- and 23-day-old infected pups were positive

regardless of the infecting dose of *Leptospira* (Table 1). The leptospire were absent in the rest of the organs except in 1 out of 2 spleens, 1 out of 4 livers, and 1 out of 4 lungs of 14-day-old pups infected with 10^8 and 10^2 doses.

Direct observation and culture of the urine of 14- and 23-day old infected pups were also performed in this study (Table 2). During the 4th week of infection, the urine of pups infected with all the doses were found to be *Leptospira*-positive. However, in the 1st week, leptospiral shedding was not yet evident in 14-day old pups infected with 10^2 and 10^4 leptospire, and 23-day old pups infected with 10^2 organisms.

3.5. Antibody production

Quantitative MAT results of sera from 14- and 23-day old infected pups revealed a seroconversion or increase in anti-K64 antibody titers from the 2nd to the 4th week of post infection (Table 3). Titers of $\geq 1:320$ were observed during the 2nd week of infection of 14-day old pups infected with 10^8 and 10^6 leptospire. However, all of the 10^4 and most of the 10^2 -infected pups were leptospire-negative. Meanwhile, in 23-day old pups infected with 10^2 leptospire, a titer as low as 1:20 was detected.

3.6. Gross and microscopic examination

At 9 days post infection all of the pups infected with 10^2 up to 10^8 leptospire showed massive hemorrhage or scattered petechiae in their lungs (Fig. 1B). The pulmonary hemorrhage, however, was not found in the lungs of the control pups.

HE staining revealed hemorrhages (black arrows) in the kidney cortex and medulla, liver, and lungs of 10^2 infected 7-day old pups, which were absent in the control (Fig. 5). In addition to hemorrhages in the kidney, congestion (blue arrows) was also found in the renal cortex.

In the liver, structural changes, especially hepatocyte disorganization, were observed in pups infected with leptospires. Inflammatory cell infiltration in the liver (red arrows) was also seen (Fig. 5).

In the cerebellum of 7 day old pups infected with 10^8 leptospires, we found hemorrhage near the blood vessel (data not shown).

Immunostaining of the spleens of infected rat pups showed an abundance of scattered *Leptospira* in the red pulp (data not shown). The presence of these bacteria was also confirmed through immunostaining of other organs such as the kidneys, livers, and lungs of 7-day-old pups infected with 10^2 leptospires (Fig. 6) and in the cerebellum of the 10^8 leptospires infected 7-day-old pups (data not shown).

4. Discussion

Previous reports on experimental leptospirosis mostly focused on acute lethal infection using guinea-pigs [7-9], hamsters [2, 6, 24], and some strains of mice [2, 5, 12-14]. Rats, which have been well known for its resistance and ability to maintain the leptospires in their kidneys, are considered as resistant animal models of leptospirosis [19]. Although it has been reported that rats with low body weight are susceptible to leptospiral infection [22], the ontogenic development of resistance remains to be elucidated.

In our study, 4-, 7-, and 14-day old were designated as the suckling age, and 23-day old as the weaning age of rat pups [22, 26]. These ages were chosen in order to determine the age at which rats become resistant to leptospiral infection. Furthermore, 10^8 , 10^6 , 10^4 and 10^2 leptospires were injected in the said pups in order to determine the leptospiral doses that make pups susceptible or resistant to infection with leptospires.

Adult rats are known to show no symptoms after *Leptospira* infection and

subsequently become persistent carriers for a certain period of time [18, 19]. In our experiment, suckling pups subcutaneously infected with different doses of leptospires showed severe illness. Jaundice, which is considered as one of the severe symptoms of leptospirosis, was observed one day prior to moribundity in 4- and 7-day old infected pups in the given doses (Fig. 1A). In 14-day old infected pups, symptoms appeared 4 days before moribundity in several pups injected with lower doses (10^4 and 10^2 leptospires). Jaundice in the rat pups was usually followed by the occurrence of neurological symptoms such as body tremors, weakness, movement disorders, and seizures. The average level of total bilirubin (Fig. 2) showed statistically significant differences in the total bilirubin level of *Leptospira*-infected pups compared to the control and between the 2 infecting doses. In an experiment performed by Higgin et al [7], jaundice was also observed one day prior to death in guinea pigs infected with *L. icterohaemorrhagiae*. They found increased levels of conjugated and unconjugated bilirubin at day 5 post inoculation, which was the same day that all the infected guinea pigs were found to be in moribund condition.

Appetite loss was apparent in the infected suckling pups. This resulted in a 5-15% decrease in body weight (Figs. 4A, B and C), which were statistically significant compared to the control ($p < 0.05$). These observations are consistent with previously reported results in hamster model of leptospirosis [27]. Twenty-three day old infected pups showed a slight decrease in body weight at 4 days post infection compared to the control (Fig. 4D), which shows no significant differences. These results showed decrease in body weight as a marker of death in susceptible rat pups.

Survival rate of infected pups revealed the correlation between the age and infecting doses (Figure 4). The doses given revealed the ability of different ages of rat pups to survive leptospiral infection. In Fig. 4A, although all of the 4-day old infected pups infected with 10^8 , 10^4 , and 10^2 doses became moribund within 5, 7, and 9 days after infection, respectively,

33.3% of pups infected with 10^6 leptospires survived infection. This phenomenon of animal being able to survive infection with lethal dose of leptospires was previously reported in a hamster infection model and is called immunization effect [28]. The failure to see mortality in some groups was possibly due to the immunization effect and further investigation would be needed. All of the 7-day old infected pups died within 4 and 7 days after infection with 10^8 and 10^4 leptospires, respectively. More survivors were observed among 14-day old infected pups compared to the younger age. We did not find any death or moribundity in the 23-day old infected pups (data not shown). These results showed an age- and dose-dependent survival of suckling and weaning rat pups against leptospiral infection. The age of rat pups at which infection commences may contribute to the prolonged time of moribundity and occurrence of symptoms found in our animal model. Our study revealed that younger age (i.e., 4- and 7-day old) of suckling rat pups are more susceptible to infection with leptospires compared to the older ones. Furthermore, higher doses of leptospires increased the susceptibility of the younger rat pups to infection. Analysis of the ontogeny of resistance of rats to leptospiral infection merits further investigation. Also, depending on the age of pups, the role of acquired (antibody production and cellular immunity) and innate immunity (phagocytes, complement system, Toll-like receptors etc.) needs elucidation.

We were able to recover leptospires in more than 50% of organ cultures from moribund infected pups (Table 1). Based on this, we were also able to verify the ability of leptospires to invade the organs of infected pups. Though rats can eliminate leptospires from all organs except kidneys [2], we were not able to observe this in the infected suckling rat pups. This may be due to the immature state of the pups' immune system. The time of moribundity (i.e., within 9 days) and recovery of leptospires found in most of the organ cultures are consistent with the dissemination of *Leptospira* through the blood stream during the leptospiremic phase [3, 4]. Thus, the suckling age of rat pups may serve as a model for

279 studying development of natural and acquired immunity to leptospires.

280 In contrast to moribund pups, surviving 14- and 23-day-old infected pups showed
281 100% positive kidney cultures (Table 1). Although 1 out of 2 spleen in 10^8 and 1 out of 4
282 livers and lungs in 10^2 -infected 14-day-old pups were leptospire-positive, our results showed
283 that as the rats enter the weaning age, its ability to clear the bacteria from the other organs,
284 except the kidneys, also increases.

285 Among the surviving rat pups, shedding of leptospires in urine begun within a week
286 after infection except for the 14-day-old infected with 10^2 and 10^4 doses, and 10^2 leptospire-
287 infected 23-day-old pups. Based on these results, higher doses of *Leptospira* seem to have
288 more potential in causing leptospiral-shedding in rat pups (Table 2). Although not all of the
289 10^2 doses showed positivity in urine by the 1st week of infection, more than half of the pups
290 shed these bacteria in their urine starting from the 2nd week of infection. These results showed
291 that the shedding potential of rat pups correspond with adults [18].

292 Quantitative MAT results of sera collected during the 2nd and 4th week of infection
293 showed at least four-fold increases relative to controls in anti-*Leptospira* (strains K64)
294 antibody titers or seroconversion in the surviving rat pups (Table 3). These results showed the
295 occurrence of acute leptospirosis in the pups. Pre-infection sera were not collected due to the
296 size of the pups and difficulty in obtaining blood. The variation of MAT titers shown in this
297 study may be due to individual differences in immune response of each rat pup since we were
298 using the outbred type of *Rattus norvegicus* species (i.e., Wistar) that may represent the
299 natural infection of *Leptospira*.

300 In this study, 14- and 23-day-old rat pups were found to shed *Leptospira* in their urine
301 in the second week after infection regardless of whether they were MAT-positive or -negative.
302 We found no relationship between antibody production and leptospiral shedding.
303 Furthermore, the 14- and 23-day-old infected pups that survived infection had *Leptospira*-

positive kidney cultures but mostly negative in other organs (Table 1), suggesting that leptospires survive in the kidney to escape from antimicrobial effects of host immunity (e.g. antibodies and phagocytes).

From the macroscopic observations (Fig. 1B), infected pups were found to have massive hemorrhage or scattered petechiae in their lungs. This was similar to what were also observed in the lungs of *Leptospira*-susceptible animal models such as hamsters [24] and humans [29].

In order to clearly observe the pathological alterations in the infected organs of rat pups, we performed HE-staining (Fig. 5). The structural changes were observed to be more pronounced in pups infected with 10^2 leptospires compared to the highest dose given (10^8 leptospires). All of the pathologic findings were also observed in a hamster infection model of acute leptospirosis [24, 30].

In the histopathological observation of the cerebellum of 10^8 leptospires infected 7-day-old pups, a small hemorrhagic focus was found near the blood vessel (data not shown). There have been reports of human brain hemorrhage due to leptospirosis in middle [31] and younger age [32]. Although hemorrhage in the central nervous system (CNS) is rare in leptospirosis [32], it has been assumed that vasculitis accompanied by disseminated endothelial destruction in the capillary may lead to intracerebral hemorrhage [30]. Neurological symptoms (weakness, tremor and seizures) that appeared among the infected suckling pups may be used to further investigate about neuroleptospirosis since, to our knowledge there are few publications on laboratory animals developing obvious CNS symptoms [33] compared to this novel *Leptospira*-susceptible rat pup model.

We also used immunohistochemistry to confirm the presence of leptospires (brown color) in each organ. All of the spleens (data not shown), kidneys, livers, and lungs (Fig.6) of 10^2 leptospire-infected pups were positive for the organisms. This finding corresponded with

the organ culture and confirmed leptospiral dissemination in infected pups' organs throughout the acute phase of leptospirosis. Also in comparison to hamsters infected with the same strain, the lungs of infected rat pups had more leptospire [24].

Pathological changes in several organs observed in this study verified the ability of *Leptospira* to cause severe leptospirosis in suckling rat pups. Moreover, the correlation between age and resistance in this novel animal model of leptospirosis may contribute to elucidating the susceptibility and resistance mechanisms of leptospiral infection in rats.

Based on these results, we propose the suckling rat pups as a novel animal model of human leptospirosis to investigate pathogenesis, development of host resistance, and the mechanisms involved in rats becoming maintenance hosts of leptospire.

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420

421 **Figure Captions**

422

423 **Fig. 1A. Jaundice was apparent in infected pup.** Jaundice in the ears, extremities, and tail
424 was evident in 7-day old rat pup infected with K64 one day prior to being moribund but not
425 in the uninfected control pup. **B. Macroscopic appearance of the lungs in 7-day-old pups.**
426 Lungs of 7-day-old infected and uninfected rat pups were excised at 9 days post infection and
427 were observed for any gross pathological changes.

428

Fig. 2. Levels of total bilirubin of 7-day old control and infected (10^2 and 10^8 leptospire) pups. The average level of total bilirubin for each dose group was determined and compared with the other groups using unpaired student t-test. P-values $p<0.001(**)$ and $p<0.05(*)$ were considered significant.

Fig. 3. Survival rate of 4-, 7-, and 14-day old pups infected with *L. interrogans* serovar Manilae strain K64. Rat pups were infected with 10^8 (—), 10^6 (—), 10^4 (—), and 10^2 (—) doses of *L. interrogans* serovar Manilae strain K64 at 4- (A), 7- (B), and 14-days after birth (C). P-values $<0.01(*)$ and $<0.05 (**)$ were considered significant.

Fig. 4. Mean body weight of 4-, 7-, 14-, and 23-day-old infected pups. Four- (A), 7- (B), 14- (C), and 23-day-old (D) rat pups were infected with *L. interrogans* serovar Manilae strain K64 at doses of 10^8 (●), 10^6 (▲), 10^4 (◆), and 10^2 (★). The mean body weight for control rat pups per dose group is also shown (■). P-values $<0.01(*)$ and $<0.05 (**)$ were considered significant.

Fig. 5. Haematoxylin-eosin staining of kidney cortex, kidney medulla, liver, and lung of control and 7-day-old pups infected with 10^2 doses of K64. Hemorrhage (black arrows) was observed in the kidney cortex and kidney medulla of pups infected with both doses. Congestions (blue arrows) were also found in the renal cortex of infected pups. In the renal medulla of infected pups, the number of collapsed lumen of the renal tubules increased compared to the control. Inflammatory infiltrates (red arrows and magnified pictures in box), erythrocyte within the sinusoids, and diffuse loss of cohesion/hepatic cord disarrangement were found in the liver of pups infected with both doses, but not in the uninfected pups (control). Enlargement of interalveolar septa in addition to hemorrhagic foci (black arrows)

454 and congestion (blue arrows) were also observed. Bars: 5 μm (100x magnification) and 1 μm
455 (400x magnification).

456

457 **Fig.6. Immunohistochemistry of the different organs of 7-day-old pups infected with 10^2**
458 **doses of K64.** Leptospire (brown color) were found in the kidneys, livers, and lungs of
459 infected pups. Bars: 1 μm .

1 **Table 1.** Results of organ culture in the 4-, 7-, 14- and 23-day-old infected and uninfected rat pups.

Infecting dose	4 day old					7 day old					14 day old				23 day old			
	Kidney	Spleen	Liver	Lung	Brain	Kidney	Spleen	Liver	Lung	Brain	Kidney	Spleen	Liver	Lung	Kidney	Spleen	Liver	Lung
Control	0/4 ^a	0/4	0/4	0/4	0/1	0/5	0/5	0/5	0/5	0/3	0/5	0/5	0/5	0/5	0/3	0/3	0/3	0/3
10 ⁸	2/3	1/3	1/3	3/4	1/1	5/6	4/6	5/6	6/6	2/2	2/2	1/2	0/2	0/2	4/4	0/4	0/4	0/4
10 ⁶	4/4	3/4	3/4	3/4	1/1	5/5	4/5	5/5	5/5	5/5	7/7	0/7	0/7	0/7	5/5	0/5	0/5	0/5
10 ⁴	5/6	4/6	4/6	6/6	2/2	6/7	6/7	6/7	8/8	4/4	2/2	0/2	0/2	0/2	5/5	0/5	0/5	0/5
10 ²	6/6	3/6	6/6	6/6	2/2	3/3	3/3	3/3	3/3	3/3	4/4	0/4	1/4	1/4	3/3	0/3	0/3	0/3

2 ^anumber of culture positive/total number of samples

- 1 **Table 2.** *Leptospira*-positive (direct microscopic observation and culture) urine of infected and uninfected 14-day-old and 23-day-old pups.

Infecting dose	14-day-old				23-day-old			
	1 st week	2 nd week	3 rd week	4 th week	1 st week	2 nd week	3 rd week	4 th week
Control	0/3 ^a	0/3	0/3	0/3	0/3	0/3	0/3	0/3
10⁸	2/2	2/2	2/2	2/2	3/4	4/4	4/4	4/4
10⁶	2/5	4/5	5/5	5/5	2/5	5/5	5/5	5/5
10⁴	NU ^b	2/2	2/2	2/2	2/5	4/5	4/5	5/5
10²	0/4	3/4	4/4	4/4	0/3	2/3	3/3	3/3

- 2 ^anumber of *Leptospira*-positive urine/total number of urine samples

- 3 ^bNU, no urine

1 **Table 3.** Quantitative MAT results of 14- and 23-day-old infected and uninfected pups.

14-day-old infected pups and control					23-day-old infected pups and control			
No.	K64		Patoc I		K64		Patoc I	
	2 week p.i.	4 week p.i.	2 week p.i.	4 week p.i.	2 week p.i.	4 week p.i.	2 week p.i.	4 week p.i.
Control	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
10 ⁸	4	1:320	1:640	-	-	NBC	1:320	-
	5	1:320	1:640	-	-	NBC	1:320	-
	6	ND	ND	ND	ND	1:640	1:2560	-
	7	ND	ND	ND	ND	1:640	1:2560	-
10 ⁶	8	1:320	1:320	-	-	-	1:160	-
	9	1:320	1:640	-	-	NBC	1:160	-
	10	1:320	1:640	-	-	-	1:160	-
	11	1:2560	1:5120	-	-	1:640	1:2560	-

	12	1:640	NBC	-	-	1:160	1:5120	-	-
10⁴	13	-	1:1280	-	-	-	1:160	-	-
	14	-	1:1280	-	-	1:80	1:160	-	-
	15	ND	ND	ND	ND	NBC	1:160	-	-
	16	ND	ND	ND	ND	1:160	1:2560	-	-
	17	ND	ND	ND	ND	1:160	1:1280	-	-
10²	18	-	1:640	-	-	1:20	1:320	-	-
	19	-	1:1280	-	-	1:160	1:80	-	-
	20	-	1:5120	-	-	1:80	1:2560	-	-
	21	1:320	1:5120	-	-	ND	ND	ND	ND

2 NBC; no blood collected due to difficulty in obtaining blood from the rat pups

3 p.i.; post infection

4 ND; not done because rat pup from this group died therefore no blood was collected

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Kidney

Liver

Lung