Characterization of *Leptospira* infection in suckling and weaning rat pups

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**A B S T R A C T**

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

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1. **Introduction**

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

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

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

Hamsters and guinea pigs have been used as susceptible animal models of leptospirosis [2,5–9]. The use of gerbils [10] and marmoset monkeys [11] in experimental leptospirosis have also been reported. Although mice are considered resistant to Leptospira 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., 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 10⁷ 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 (1966) showing rat body weight limits in fatal cases of Leptospira 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 leptospliral infection in suckling and weaning rat pups.

2. Materials and methods

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. 4-, 7-, 14-, and 23-day-old pups were then subcutaneously infected with 10⁴, 10⁵, 10⁶, and 10⁷ organisms of L. interrogans serovar Manilae strain K64 [23,24]. Low passage (<10×) in vitro sub-cultured organisms were suspended in PBS and used for infection [23] (see Section 3.2 for the number of pups per group). Pups injected with PBS only were used as controls (uninfected). The pups were monitored daily for their body weight and clinical signs of illness such as loss of appetite, decreased or loss of activity, etc. Pups showing continuous weight loss, jaundice, and loss of activity were considered to be moribund, and were euthanized by inhalation of sevoflurane (Sevoflurane, Maruishi Pharmaceutical Co., Ltd, Osaka, Japan). Infected and uninfected rat pups were observed until 30 days post bacterial inoculation.

2.2. Total bilirubin

To measure the amount of total bilirubin in sera, the blood was aseptically collected by cardiac puncture of moribund 7-day-old pups injected with 10² and 10⁸ leptospires (lowest and highest doses) as well as uninfected pups. Total bilirubin in serum was measured by using Bilirubin Kit-K (Al fresco Pharma Corporation, Osaka, Japan). This was done by mixing 20 μl of each serum sample with Diphyl line, Diazio and Fehling reagents (200 μl per reagent) and color reaction was measured using spectrophotometer (600 nm).

2.3. Organ culture

Kidneys, spleens, livers, lungs, and brains were collected from 4- and 7-day-old infected pups that became moribund after infection. These organs were aseptically removed, placed in disposable 2.5 ml syringe, and crushed into 4 ml sterile Korthof’s medium with 5-fluorouracil (5-FU; 100 μg/ml), and incubated at 30 °C [23,24]. The next day, 500 μl of the culture supernatant was sub-cultured in fresh Korthof’s medium and further incubated. The presence or absence of Leptospira in the cultures was observed until 60 days.

2.4. Urine

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

2.5. Quantitative microscopic agglutination test (MAT)

Whole blood was collected from the retro-orbital plexus of surviving 14- and 23-day-old infected pups during the 2nd and 4th week post infection. Serum was obtained by centrifuging the whole blood at 3000 rpm for 30 min. The serum samples were then heat inactivated for 30 min at 56 °C, cooled down, and stored at −20 °C until MAT was performed.

MAT was done using pathogenic L. interrogans serovar Manila strains K64 and saprophytic L. biflexa strain Patoc I as antigens to detect the presence of anti-Leptospira antibodies and their titers in the sera of rat pups. MAT was carried out using the quantitative method specified in the World Health Organization (WHO)-International Leptospirosis Society (ILS) Guidance on Human Leptospirosis [25]. Titers greater than or equal to 1:20 were considered significant (MAT-positive) [23].
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 µm 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) leptospires, 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).
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 leptospires were absent in the rest of the organs except in 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 leptospires, 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 ≥1:320 were observed during the 2nd week of infection of 14-day old pups infected with 10^2 and 10^6 leptospires. However, all of the 10^2 and most of the 10^2-infected pups were MAT-negative. Meanwhile, in 23-day old pups infected with 10^2 leptospires, 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 10^6 leptospires 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

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**Fig. 2.** Levels of total bilirubin of 7-day old control and infected (10^2 and 10^4 leptospires) 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.

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 (Fig. 4A and B). 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 leptospires 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

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**Fig. 3.** Survival rate of 4-, 7-, and 14-day old pups infected with L. interrogens serovar Manilae strain K64. Rat pups were infected with 10^8 ( ), 10^5 ( ), 10^4 ( ), and 10^2 ( ) doses of L. interrogens serovar Manilae strain K64 at 4- (A), 7- (B), and 14-days after birth (C), p-Values ** p < 0.001 and * p < 0.05 were considered significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
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.

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

<table>
<thead>
<tr>
<th>Infecting dose</th>
<th>4 Day old</th>
<th>7 Day old</th>
<th>14 Day old</th>
<th>23 Day old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kidney</td>
<td>Spleen</td>
<td>Liver</td>
<td>Lung Brain</td>
</tr>
<tr>
<td>Control</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/1</td>
</tr>
<tr>
<td>$10^8$</td>
<td>2/3</td>
<td>1/3</td>
<td>3/4</td>
<td>1/1</td>
</tr>
<tr>
<td>$10^6$</td>
<td>4/4</td>
<td>3/4</td>
<td>3/4</td>
<td>1/1</td>
</tr>
<tr>
<td>$10^4$</td>
<td>5/6</td>
<td>4/6</td>
<td>6/6</td>
<td>2/2</td>
</tr>
</tbody>
</table>

* Number of culture positive/total number of samples.
### Table 2

<table>
<thead>
<tr>
<th>Infecting dose</th>
<th>14-Day-old</th>
<th>23-Day-old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st week</td>
<td>2nd week</td>
</tr>
<tr>
<td>Control</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>10⁶</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>10⁴</td>
<td>2/5</td>
<td>4/5</td>
</tr>
<tr>
<td>10²</td>
<td>NU b</td>
<td>2/2</td>
</tr>
<tr>
<td>0</td>
<td>0/4</td>
<td>3/4</td>
</tr>
</tbody>
</table>

* Numbers of *Leptospira*-positive urine/total number of urine samples.

b NU, no urine.

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**Fig. 5.** Haematoxylin-eosin staining of kidney cortex, kidney medulla, liver, and lung of control and 7-day-old pups infected with 10² 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) and congestion (blue arrows) were also observed. Bars: 5 µm (100× magnification) and 1 µm (400× magnification). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
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, $10^8$, $10^5$, $10^4$ 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

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

<table>
<thead>
<tr>
<th>No.</th>
<th>14-Day-old infected pups and control</th>
<th>23-Day-old infected pups and control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K64</td>
<td>Patoc I</td>
</tr>
<tr>
<td></td>
<td>2 Week p.i. 4 Week p.i.</td>
<td>2 Week p.i. 4 Week p.i.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>1:320</td>
<td>1:640</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>1:320</td>
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<td>8</td>
<td>1:320</td>
<td>1:640</td>
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</tr>
<tr>
<td>21</td>
<td>1:320</td>
<td>1:5120</td>
</tr>
</tbody>
</table>

NBC: no blood collected due to difficulty in obtaining blood from the rat pups, p.i.: post infection and ND: not done because rat pup from this group died therefore no blood was collected.

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**Fig. 6. Immunohistochemistry of the different organs of 7-day-old pups infected with $10^2$ doses of K64.** Leptospires (brown color) were found in the kidneys, livers, and lungs of infected pups. Bars: 1 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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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 (Fig. 4A-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 (Fig. 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 $10^4$ 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 studying development of natural and acquired immunity to leptospires.

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

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

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

In this study, 14- and 23-day-old rat pups were found to shed *Leptospira* in their urine in the second week after infection regardless of whether they were MAT-positive or -negative. We found no relationship between antibody production and leptosomal shedding. 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 leptospires [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 leptospirosis 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 leptospiroses.

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