High virulence in hamsters of four dominant Leptospira serovars isolated from rats in the Philippines

Sharon Y. A. M. Villanueva,1 Mitsumasa Saito,1 Yutaka Tsutsumi,2 Takaya Segawa,1 Rubelia A. Baterna,3 Antara Chakraborty,1 Tatsuma Asoh,1 Satoshi Miyahara,1 Yasutake Yanaigihara,3 Lolita L. Cavinta,3 Nina G. Gloriani3 and Shin-ichi Yoshida1

Correspondence
Sharon Y. A. M. Villanueva
svillan@bact.med.kyushu-u.ac.jp

1Department of Bacteriology, Faculty of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan
2Department of Pathology, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan
3Department of Medical Microbiology, College of Public Health, University of the Philippines-Manila, Manila 1000, Philippines

Leptospirosis is caused by pathogenic species of Leptospira. The aim of this study was to determine and characterize the pathogenicity of four dominant Leptospira isolates prevailing among rats in the Philippines. The isolates were Leptospira interrogans serovar Manilae strain K64, L. interrogans serovar Losbanos strain K37, L. interrogans serovar Ratnapura strain K5 and Leptospira borgpetersenii serovar Javanica strain K6. Pathogenicities were studied using hamsters, which reproduce severe human leptospirosis. The minimum lethal doses were 10^5 (±1) leptospires for K64, K37 and K5, and 10^1 leptospires for K6. Weight loss amongst the Leptospira-infected hamsters was observed from 1 day before death (K64-, K37- and K5-infected hamsters) to as much as 1 week before death for K6-infected hamsters. Similar and varied gross and microscopic lesions were observed amongst infected hamsters, even for strains belonging to the same species (i.e. L. interrogans). The most significant and common histopathological findings were congestion of the glomerulus, disarrangement of hepatic cords and erythrophagocytosis. Other findings were foamy splenic macrophages for K6, severe petechial pulmonary haemorrhage for K64, and hematuria and severe pulmonary congestion for K37. Immunostaining and culture revealed the presence of leptospires in different organs of the infected hamsters. Based on these results, Leptospira isolates from rats in the Philippines were shown to be highly virulent, causing pulmonary haemorrhage, severe hepato-renal damage and death in hamsters even at lower doses. The present findings on experimental leptospirosis support clinical data showing that patients with severe manifestations of leptospirosis, such as pulmonary haemorrhage, are increasing in the Philippines. These findings may serve as a basis to strengthen the early diagnosis and treatment of human leptospirosis.

INTRODUCTION

Leptospirosis is a zoonotic infection caused by pathogenic spirochaetes belonging to the genus Leptospira. It is distributed widely around the world, especially in countries with tropical or subtropical climates (WHO & ILS, 2003). It is the leading cause of zoonoses worldwide (WHO, 1999; Levett, 2001; Vinetz, 2001). Manifestations of leptospirosis in humans vary from mild, flu-like symptoms to severe manifestations causing multi-organ failure, particularly renal, hepatic and sometimes pulmonary failure, and even death (WHO & ILS, 2003; Evangelista & Coburn, 2010; Faine et al., 1999; Kobayashi, 2005). Animals, however, either may be susceptible and
exhibit similar symptoms to humans or may be carriers of the organisms and not manifest any signs of illness, e.g. rodents (especially rats) (WHO & ILS, 2003; Levett, 2001).

In 1916, Inada and colleagues reported the discovery of the causative agent of Weil’s disease (Inada et al., 1916), which was then named Spirochaeta icterohaemorrhagica. These researchers, who were then affiliated with the First Medical Clinic of the Imperial University in Kyushu (currently known as Kyushu University), Japan, were interested in Weil’s disease due to the epidemic that occurred in Japan wherein patients, mostly coal miners, presented with muscular pain, jaundice, fever, conjunctival congestion, haemorrhage and albuminuria. They were able to isolate leptospires from the livers of guinea pigs injected with the blood of a Weil’s disease patient and concluded that these organisms were the causative agent of Weil’s disease.

There have been few publications regarding clinical presentations of leptospirosis among patients in the Philippines. Filipinos suspected of having leptospirosis were reported to have presented with a wide spectrum of signs and symptoms, such as fever, headache, jaundice, conjunctival suffusion, abdominal pain, myalgia, oligaemia and or/ proteinuria etc. (Amlasan et al., 2012; Yanagihara et al., 2007). Yanagihara et al. (2007) also reported that the mortality rate of leptospirosis, although unofficial, in two major hospitals in the Philippines was 12–14 %, and that the major cause of death was renal failure. However, in the study by Amilasan et al. (2012), the leading cause of death in 18 out of 51 leptospirosis patients in San Lazaro Hospital in the Philippines in 2009 was pulmonary haemorrhage. This was followed by acute respiratory distress syndrome/severe respiratory failure, acute renal failure and, finally, multiple organ failure. Recently, there have been unofficial reports of a seemingly increasing number of cases of pulmonary haemorrhage among leptospirosis patients in the Philippines. Outbreaks of severe pulmonary haemorrhage were also reported in Nicaragua (Trevejo et al., 1998), Brazil (Gouveia et al., 2008) and other countries (McBride et al., 2005).

Rodents, especially rats, are considered to be the most significant reservoir of leptospires. Hideo Noguchi first isolated leptospires from a Norwegian rat in the USA (Noguchi, 1917). Rats became infected with Leptospira but did not become sick or die from the infection. In 2010, we reported the isolation of leptospires from the kidneys of rats in the Philippines (Villanueva et al., 2010). These isolates were classified previously into four groups, Leptospira interrogans serovar Manilae, L. interrogans serovar Losbanos, L. interrogans serovar Ratnapura and Leptospira borgpetersenii serovar Javanica, based on leptospiral gyrase B encoding gene (gyrB) sequence analysis and PFGE. These four groups were found to be the major circulating leptospires in the Philippines and were, therefore, used in the current study. Although there have been several reports on the isolation of leptospires from the Philippines, most were isolations from rats. There is little known regarding isolations of leptospires from human leptospirosis cases. Furthermore, there are still no publications on the characterization, in terms of pathogenicity (especially using animal models), of the isolates in the Philippines. Our study, therefore, aimed to characterize four Leptospira isolates, representing each of the four dominant serovars from the Philippines, by performing pathogenicity tests on golden Syrian hamsters (Mesocricetus auratus).

Studies on infectious diseases mostly use non-human models, especially laboratory rodent models (Salyers & Whitt, 2002). Laboratory rodents are usually used because they are small, are relatively cheap and can be cared for easily. Animal models for human infection, ideally, would develop symptoms and bacterial distribution in the body that mimics the human form of infection. Golden Syrian hamsters are highly susceptible to leptospiral infection and are considered to be good animal models for leptospirosis (Haake, 2006). This animal is preferred in most leptospirosis research because the symptoms and severity of infection in hamsters are similar to those observed in humans.

In this paper, we report that hamsters infected with the four Leptospira isolates from the Philippines showed common and varying patterns of pathogenicity in terms of gross and microscopic lesions, weight loss, clinical presentations, etc. The most important finding of this study was the demonstration of the high virulence of all four of these Leptospira strains since they caused death in hamsters even when infected with low doses (i.e. 1–10 leptospires).

METHODS

Leptospira strains used in hamster infection. In our previous study (Villanueva et al., 2010), 10^5 and 10^3 leptospires isolated from rat kidneys were intraperitoneally injected into golden Syrian hamsters and were observed for 21 days. Most of the isolates proved lethal to hamsters. For our current study, one strain from each Leptospira serovar dominant amongst rats was chosen. These were L. interrogans serovar Manilae strain K64, L. interrogans serovar Losbanos strain K37, L. interrogans serovar Ratnapura strain K5 and L. borgpetersenii serovar Javanica strain K6. These four strains were chosen because of their supposed high virulence (i.e. causing death in hamsters <1 week after infection). Prior to infection, these strains were subcultured in modified Korthof’s medium and were grown to confluency (4–6 days old; ~10^8 leptospires ml^-1) at 30°C. Motile leptospires were enumerated under a dark-field microscope using a Thoma counting chamber. The bacterial solutions were then diluted serially (10-fold) until the doses used for infection were reached (see below).

We hypothesized that leptospires in the environment are present in small doses or are highly diluted in surface water, etc. Therefore, we carried out our experiment by infecting hamsters with 1 ml low-passage (<10 times in vitro subcultures) leptospires at low doses, e.g. 10^1, 10^2, 10^3 and 10^6 leptospires.

When using animal models of disease, the infection route should be similar to the natural route of infection in humans (Salyers & Whitt, 2002). Since leptospires are known to infect humans and susceptible animals via the mucous membrane or skin (WHO & ILS, 2003;
Levett, 2004), we subcutaneously injected the leptospires to mimic the natural route of infection.

**Production of antiserum in rabbits.** Polyclonal antibodies against *L. interrogans* serovar Manilae (strain LT 398), *L. interrogans* serovar Losbanos (strain LT 101-69) and *L. interrogans* serovar Icterohaemorrhagiae (strain Icterohaemorrhagiae no. 1) were raised in female Japanese white rabbits according to the World Health Organization and International Leptospirosis Society guidance on human leptospirosis (WHO & ILS, 2003). Briefly, the rabbits were inoculated with 5- to 7-day-old cultures of ~1 × 10^5 live leptospires through the marginal ear vein at 7 day intervals until a titre of at least 1:12 800 was reached. The rabbits were then exsanguinated through cardiocentesis, and their sera were separated and filtered. The sera were then aliquoted in 1 ml volumes and kept frozen at −80 °C until further use. Sera were heat inactivated at 56 °C for 30 min prior to use for immunostaining.

**Pathogenicity tests in hamsters.** Four-week-old male golden Syrian hamsters were randomly assigned to groups of five hamsters. After 1 week of acclimatization to the environment (i.e. the animal care facility), the hamsters were infected with the bacteria as described above. Negative control (culture media-injected/uninfected) hamsters were subcutaneously injected with 1 ml Korthof’s medium only. Hamster food and water were given ad libitum. The infected and negative control hamsters were observed two or three times a day for up to 28 days. Daily body weight monitoring was done and hamsters were observed for any signs of clinical illness, such as prostration, excitability, isolation, decreased food and water intake, pilo erection/ruffled fur, external haemorrhage, etc. (Oliva *et al.*, 1994). Moribund hamsters were sacrificed by inhalation of sevoflurane and cervical dislocation. Hamsters surviving after day 28 of infection were also sacrificed in the same manner as the moribund hamsters. Survival and body weight monitoring results were representative of two independent experiments.

**Isolation of leptospires from hamster tissues**

*Whole blood and urine.* One drop of whole blood and, if available, urine were cultured in separate tubes containing 4 ml modified Korthof’s medium with 5-fluorouracil (5-FU; 100 μg ml⁻¹). 5-FU was added to the culture medium in order to prevent the growth of contaminants (WHO & ILS, 2003). The tubes were then incubated at 30 °C and observed weekly for 1 month.

*Kidney, liver, lung and spleen.* One kidney and parts of the liver, lungs and spleen were macerated using a sterile syringe, transferred to the 5-FU-containing modified Korthof’s medium, and then incubated at 30 °C. The next day, 500 μl supernatant from each cultured organ was transferred to fresh medium without 5-FU, further incubated at the same temperature and observed weekly for 1 month.

**Gross and microscopic examination of organs**

Upon dissection, hamster organs were observed for any pathological changes (i.e. colour changes, presence or absence of haemorrhages, congestion, etc.). For microscopic examination, slices of the kidney, liver, lungs and spleen were fixed in 20% neutral buffered formalin for >1 month. Pathologists who were blind to the information regarding the organs and the associated strains did the scoring of the histopathological sections. More than three sections were reviewed and representative sections are shown in the figures.

**Haematoxylin–eosin (HE) and periodic acid–Schiff (PAS) staining.** Formalin-fixed tissues were embedded in paraffin, cut into thin sections (~4 μm), and stained with HE and PAS as routinely carried out.

**Immunostaining.** During preliminary experiments, anti-Manilae antiserum gave the best results in immunostaining compared with anti-Losbanos and anti-Icterohaemorrhagiae antisera. This antiserum was therefore used in the immunostaining procedures of different organs at 1:500 dilutions. Endogenous peroxidase activity in tissue sections of infected and uninfected hamsters was inactivated by soaking them in 0.3% hydrogen peroxide in ethanol for 20 min. The amino acid polymer method (Simple Stain; Nichirei) was employed to visualize the antigen localization (in brown) with the diaminobenzidine reaction. The nuclei were counterstained with haematoxylin. No antigen retrieval step was used since in a preliminary study, protease pretreatment or hydrated heating treatment in citrate buffer did not strengthen the reaction product. As a control, commercially available rabbit antiserum against *Treponema pallidum* (Biocare Medical) was used at a dilution of 1:500.

**Animal ethics.** All animal experiments were reviewed and approved by the Ethics Committee on Animal Experiment at the Faculty of Medical Sciences, Kyushu University (permit no. A24–141–0). The experiments were also carried out under the conditions stipulated in the Regulations for Animal Experiments of Kyushu University.

## RESULTS

**Body weight monitoring, clinical signs and survival analysis**

Infected and uninfected (negative control) hamsters were monitored daily for any signs and symptoms of leptospirosis, and for any changes in body weight. Uninfected hamsters did not manifest any signs and symptoms of illness. However, hamsters infected with strains K64, K37 and K5 were observed to lose weight from 1 to 4 days prior to death, regardless of the infecting dose (Fig. 1a–c, upper panel). The mean body weight loss of hamsters infected with these three strains prior to death ranged from 7 to 14 %. However, for K6-infected hamsters, weight loss was observed for ~4–5 days prior to death (Fig. 1d, upper panel) with a mean range of 23–31 %. Negative control hamsters, however, had a steady weight increase (mean of 64 %) until day 28 of the observation period (Fig. 1a–d, upper panels).

Hamsters infected with strain K64 died the fastest, at 8 days post-infection (Fig. 1a, lower panel), whilst those infected with strain K6 started dying at 13 days post-infection (Fig. 1d, lower panel). Doses of strains K64, K37 and K5 as low as 10^6 (=1) leptospires were able to cause death among hamsters (Fig. 1a–c, lower panels). However, for K6-infected hamsters, the lowest lethal dose was found to be 10^1 (Fig. 1d, lower panel). The LD₅₀ was 10^6 leptospires ml⁻¹ for strains K64, K37 and K5, and 5 × 10^6 (=5) leptospires for strain K6.

The following signs were observed in most of the infected hamsters regardless of the infecting strains: excitability, isolation, pilo erection/ruffled fur, hunched back, prostration, decreased water and food intake, decreased activity or movement, and closing of eyes/conjunctivitis (i.e. presence of ocular discharge). Some infected hamsters were also observed to have body tremors. These signs were mostly seen 1 day before death in the infected hamsters. In addition, all of the infected hamsters, except for K6-infected hamsters, were found to be jaundiced.
Isolation of leptospires from the organs, blood and urine of infected hamsters

Leptospires were recovered from most of the organs, blood and urine of hamsters infected with strains K64, K37 and K5, regardless of infecting dose, revealing a moribund systemic infection. However, for K6-infected hamsters, leptospires were recovered from the kidneys of only two hamsters infected with a dose of $10^1$ leptospires. Leptospires were recovered from the blood and kidney of one of the two hamsters that survived infection with $10^0$ leptospires of strain K37. Uninfected (negative control) hamster organs, blood and urine were negative for leptospires.
Gross and microscopic examination of organs

The organs of moribund and surviving hamsters were examined macroscopically and microscopically. For microscopic examination of organs, HE staining and immuno-staining were performed. The gross and microscopic examinations of the hamster organs revealed similarities and differences in the hamsters infected with the four *Leptospira* strains isolated from the Philippines (as shown in Table 1, Figs 2, 3, 4 and 5). Immunostaining revealed the presence of leptospires in the different organs of leptospire-infected hamsters, but not in the negative control hamsters (Figs 2, 3 and 5). Immunostaining with *T. pallidum* antiserum consistently served as a negative control (data not shown). Leptospires in sections were found to be in spiral forms in intercellular spaces, but appeared to be in brown granular patterns when the pathogens were seen inside the cell.

Kidney. On gross examination, leptospire-infected hamster kidneys were found to be either dark red (almost black) or with petechial haemorrhages (Fig. 2d). Shrunken and wrinkled or raisin-like kidneys were also observed in one of five hamsters each from K37-infected (10⁰ leptospires) hamsters and K6-infected (10³ leptospires) hamsters.

Microscopically, the kidneys of the infected hamsters were shown to have glomerular congestion and renal tubular damage (Table 1, Fig. 2b, c). There were mild luminal dilatations of proximal convoluted tubules, prominent Golgi areas of proximal convoluted tubular epithelial cells and derangement of the distal convoluted tubular epithelial cells. However, only a few inflammatory cells were observed. Hyaline casts were often observed in the kidneys of infected hamsters. There were no gross and histopathological changes observed in the kidneys of uninfected hamsters (Fig. 2a, d, ‘uninfected’). PAS staining was also performed on the infected and uninfected hamster kidneys. However, no renal tubular changes were observed beyond those that were detected using HE staining (data not shown). There were also no leptomysal cells observed using PAS staining.

Immunostaining of the kidneys of infected hamsters revealed that spiral-shaped leptospires were detected in the intercellular spaces of renal tubules. The pathogens were also observed as brown granules in the cytoplasm of the renal tubular epithelial cells (Table 1, Fig. 2e, f). No pathogens were detected in the kidneys of uninfected hamsters.

Hamsters infected with strain K37 were found to have haematuria in their urinary bladders, whilst those hamsters infected with strains K64, K5 and K6 had clear yellow urine. Also, some of the K37-infected hamsters had blood on the openings of their urethras. The urine of the control hamsters, however, remained turbid or milky white.

Table 1. Summary of major findings observed in hamsters infected with four *Leptospira* serovars

<table>
<thead>
<tr>
<th></th>
<th><em>L. interrogans</em> sv. Manilae strain K64</th>
<th><em>L. interrogans</em> sv. Losbanos strain K37</th>
<th><em>L. interrogans</em> sv. Ratnapura strain K5</th>
<th><em>L. borgpetersenii</em> sv. Javanica strain K6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum lethal dose</td>
<td>10⁰</td>
<td>10⁰</td>
<td>10⁰</td>
<td>10¹</td>
</tr>
<tr>
<td>Pathological observations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congestion in glomerulus</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal cell damage</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunostaining of leptospires</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disarrangement of hepatic cell cords</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congestion of lobule</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increase in number of Kupffer cells</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunostaining of leptospires</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemorrhage</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congestive oedema</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alveolar collapse</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunostaining of leptospires</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congestion of red pulp</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shrinkage of white pulp</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foamy macrophages</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrophagocytosis</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunostaining of leptospires</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

++, Very strong; +, strong; ±, weak, −, absent.
Liver. The livers of infected hamsters were found to be congested upon gross examination. HE staining of the livers of infected hamsters revealed swollen hepatocytes, intermediate congestion of liver lobules and an increase in the number of Kupffer cells (Table 1, Fig. 3b). However, there were fewer cellular infiltrates observed in the portal vein of these hamsters. There were also disarrangements of hepatic cell cords. The hepatic cell damage was observed to be more severe among K64- and K37-infected hamsters compared with the other two strains.

The presence of leptospires among the hepatocytes of infected hamsters was confirmed by immunostaining (Fig. 3c). Leptospires were found mainly in intercellular spaces. Kupffer cells phagocytosed the leptospires and were observed as brown intracellular granules. Furthermore, some hepatocytes also showed granular cytoplasmic positivity.

Lung. The lungs of strains K64-infected hamsters were observed to have petechial haemorrhage, whilst those of K37-, K5- and K6-infected hamsters had congestive oedema (Fig. 4b). Alveolar collapse and microhaemorrhage were observed in the lungs of infected hamsters, but not in the uninfected group (Fig. 4a, c–f).

Immunostaining revealed the presence of granular-formed leptospires mainly in the cytoplasm of alveolar macrophages of infected hamsters, but not in the negative control group (data not shown). However, compared with the other organs, relatively few leptospires were detected in the lungs of infected hamsters.

Spleen. The white pulp of the spleen of all infected hamsters was observed to have shrunken (Table 1, Fig. 5b, e). Furthermore, the red pulp of the spleen of infected hamsters, except K6-infected hamsters, showed marked congestion with prominent erythrophagocytosis by macrophages (Fig. 5b, e). Interestingly, a unique phenomenon observed only in K6-infected hamsters was the presence of foamy macrophages with infrequent erythrophagocytosis (Fig. 5e, f).

Immunohistochemistry results revealed that leptospires were abundant in the erythrophagocytic or foamy macrophages in the red pulp of the spleen of infected hamsters.
DISCUSSION

This is to the best of our knowledge the first extensive report on the characterization of the pathogenicity of *Leptospira* isolates from rat kidneys in the Philippines, which were found to be highly virulent since they were able to cause death in hamsters even at a very low dose of $10^0$ (=1) leptospires (Table 1, Fig. 1). The results of our current study were similar to the results of Silva et al. (2008), wherein *Leptospira* isolates from Brazil also proved lethal even at a very low dose of $10^1$ leptospires. It is well known that humans and animals usually acquire leptospirosis through direct contact with infected animals or from an environment contaminated with the urine of infected animals (WHO & ILS, 2003). However, until now, the amount of leptospires present in the environment and infecting susceptible hosts has not been well elucidated. We hypothesized that leptospires in the environment (e.g. flood, soil, etc.) are present at low densities or are highly diluted, especially when flooding occurs. Based on this hypothesis, we inoculated small doses of four isolates into golden Syrian hamsters and observed the effects on these animals in terms of their survival or development of disease. The results of our study suggest strongly that leptospires present in contaminated environments, which humans and other susceptible animals are exposed to, may be present at low doses, but are still sufficient to successfully cause infection and even death.

As the severe type of leptospirosis in humans is reproduced in golden Syrian hamsters, our results suggest the gravity of human leptospirosis in the Philippines caused by leptospire transmission from rats. It is important to note that similar and varied findings were seen in the various macroscopic and microscopic lesions of hamsters caused by leptospires belonging to the same species, but different serovars (i.e. *L. interrogans* serovar Manilae strain K64, *L. interrogans* serovar Losbanos strain K37 and *L. interrogans* serovar Ratnapura strain K5) (Table 1, Figs 2, 3, 4 and 5). Severe damage to tubular epithelial and hepatic cells was observed amongst K64- and K37-infected hamsters, whilst less severe damage was seen in K6- and K5-infected hamsters. Furthermore, formation of hyaline casts in the renal tubules was seen mostly in K64- and K37-infected hamsters, suggesting invasion of leptospires probably from blood vessels to the renal tubules, through the damaged epithelium.

The mean weight loss prior to death of hamsters infected with strains K64, K37 and K5, regardless of infecting dose, ranged from 7 to 12%. However, K6-infected hamsters had the highest weight loss prior to death, at 31% for $10^3$ leptospires, 12% for $10^2$ leptospires and 24% for $10^1$ leptospires. However, all hamsters infected with the lowest dose (100 leptospires) of K6 survived the infection and had a 24% weight increase. In a study by Coutinho et al. (2011), the authors stressed the importance of monitoring the infected animal’s body weight, as well as other clinical parameters, since different bacterial doses and strains may likely present different disease patterns, which were
observed in our current study. They also observed that a 10% weight loss was an effective determinant of a premorbid condition in leptospire-infected animals. However, in our hamster models, it was quite difficult to use this criterion as we observed that K64-, K37- and K5-infected hamsters had slightly lower weight losses than those reported, but died suddenly shortly after they were observed to be active and without obvious signs of illness. Furthermore, the weight loss of K6-infected hamsters was greater (12–31%) than that recommended by Coutinho et al. (2011).

Amongst the four strains used in this study, hamsters infected with strain K6 were observed to take the longest time to die and had continuous weight loss, and all of those infected with $10^6$ leptospires of this strain were found to have recovered from infection (Fig. 1). Hamsters infected with this strain were the only group that did not have jaundice, but had foamy macrophages in their spleen. The foamy (pauci-erythrophagocytic) macrophages observed in the red pulp of the spleen of K6-infected hamsters were similar to those observed by Nally et al. (2004), wherein the spleens of guinea pigs infected with strains RJ15958 or RJ16441 had enlarged foamy macrophages. Foamy macrophages have always been associated with *Mycobacterium tuberculosis* (Russell et al., 2009), and other infections caused by *Toxoplasma gondii* (Portugal et al., 2008) and *Chlamydia pneumoniae* (Kalayoglu & Byrne, 1998). Formation of this type of macrophage is thought to be due to an inflammatory response or due to the nutritional requirements of pathogens. In K6-infected hamsters, leptospires may have taken advantage of the development of foamy macrophages in order to thrive in the host’s body for a long time, evade the immune system and cause prolonged systemic infection. In addition, leptospires were not isolated from most of the organs, blood or urine of K6-infected hamsters. These observations in strain K6 merit further investigation because of the uniqueness of this strain compared with the other three strains used.

Haemophagocytosis, particularly secondary or reactive haemophagocytic syndrome, is often associated with severe viral (e.g. herpes virus, human immunodeficiency virus, hepatitis, influenza, Epstein–Barr virus, etc.) and bacterial infections (e.g. superantigen-related disorders such as streptococcal toxic shock syndrome, spiroachetal infection and mycobacterial infection) (Fisman, 2000; Kuriyama et al., 2012; Roupahael et al., 2007; Silva-Herzog & Detweiler, 2008). It is an indication of the activation of
macrophages, which is commonly associated with hypercytokinaemia (also known as a cytokine storm). During a cytokine storm, cytokines are actively secreted, and may cause high fever, swelling and redness, extreme fatigue and nausea, and may also be lethal. Overexpression of cytokines and chemokines observed in hypercytokinaemia had been thought to be associated with severe organ lesions and poor prognosis (i.e. kidney, liver and lung dysfunction, bleeding, and higher mortality) amongst humans (Tajiki & Salomão, 1996; Wagenaar et al., 2009) and susceptible animal hosts, such as hamsters (Matsui et al., 2011; Vernel-Pauillac & Goarant, 2010), with leptospirosis. In our study, erythrophagocytosis by activated macrophages in the splenic red pulp was characteristic in K64-, K37- and K5-infected hamsters. The diffuse distribution of phagocytic macrophages in the splenic red pulp of infected hamsters strongly suggests a certain systemic phenomenon. Inada et al. (1916) also observed this phenomenon almost a century ago when they infected guinea pigs with the blood of a leptospirosis patient. The results of our study suggest that the activation of splenic macrophages or Kupffer cells in the liver causes the tissue injuries observed in infected hamsters. It should be noted that there was little neutrophilic or lymphocytic infiltration in the infected tissue, and this phenomenon has been reported repeatedly as one of the histopathological features of leptospirosis (Feigin et al., 1975). Activation of macrophages without association of inflammatory reactions is thus quite unique in leptospirosis. Bacterial LPS, which is abundant on the leptospiral surface, contributes to the activation of macrophages. However, it has often been reported that the endotoxicity of leptospiral LPS is lower compared with that of other Gram-negative bacteria (Isogai et al., 1986; Shimizu et al., 1987).

Pulmonary haemorrhage and/or congestion observed in the infected hamsters, particularly in K64- and K37-infected hamsters, should serve as a warning, especially among health professionals dealing with leptospirosis patients. L. interrogans serovars Manilae and Losbanos, of which these two strains belong, are the two most commonly circulating serovars in rats and humans in the Philippines (Villanueva et al., 2010; unpublished data). In 1974, Miller and colleagues reported that hamsters infected with L. interrogans serovar Icterohaemorrhagiae strain SC 2165 had ecchymotic lungs with moderate to severe congestion and were also covered with multiple, dark red petechiae (Miller et al., 1974), which was similar to what we observed in our infected hamsters. Although culture results were positive, immunostaining results of the hamster lungs in our study

Fig. 5. Microscopic examinations of the spleens of infected and uninfected hamsters. (a, b, c, e, f) HE staining of the spleens of uninfected (a), K37-infected (b, c) and K6-infected (e, f) hamsters obtained on days 28, 13 and 16 post-infection, respectively. (c) Enlarged portion of the boxed area of (b) showing erythrophagocytosis. (f) Enlarged portion of the boxed area of (e) indicating foamy macrophages. (d) Immunostaining of the spleen of a K6-infected hamster. The infectious dose was 10^2 leptospires for both K37 and K6. Bars, 50 μm (a and d), 10 μm (b, e) and 20 μm (c, f).
revealed very few leptospires (data not shown). Again, this was similar to what was observed by Miller et al. (1974) in their study. They also noted that as post-infection time increases, pulmonary haemorrhage became more severe, but the leptospiral count increased only slightly. It was suggested that the severity of pulmonary haemorrhage was not related directly to the leptospiral activity in the lungs, but may be a consequence of certain ‘toxic substances’ supposedly released by Leptospira in vivo in other organs, such as the liver (Miller et al., 1974; Arean et al., 1964). Pulmonary haemorrhage, sometimes leading to death, among Filipinos with leptospirosis as well as patients in other countries has become a trend in recent years. Knowing the gravity of infection caused by the strains circulating should help doctors and health practitioners to administer prompt diagnosis and treatment to prevent progression of the disease to a severe state.

The presence of leptospires in the blood, urine and different organs was confirmed through the recovery of leptospires in the cultures as well as by immunostaining of the different organs of the infected hamsters (Figs 2, 3 and 5). Leptospires were not recovered in most of the organs, blood and urine of K6-infected hamsters, but immunostaining proved the presence of organisms in the organs of these hamsters. However, compared with the other three strains, immunostaining revealed few leptospires in the organs of K6-infected hamsters. In this study, immunostaining revealed the presence of leptospires as brown granular debris when they were located or phagocytosed in the cytoplasm, or as brown spiral-shaped organisms in intercellular spaces. These observations are similar to previous reports by Nally et al. (2004) on guinea pig organs and De Brito et al. (2006) on the organs of leptospirosis patients and experimentally infected guinea pigs. The presence of leptospires in the intercellular spaces of the kidney and liver-in infected hamsters observed in our current study suggests the inaccessibility of the inflammatory and immune cells to the pathogens. This may be one of their immune ‘evasion mechanisms’, in order to survive and probably multiply in the those organs. Also, the kidney and liver preference of leptospires may be attributed to the fact that these organs have an abundant supply of lipids that contain fatty acids, which are essential for leptospiral growth (Baseman & Cox, 1969; Stern et al., 1969).

Whether the different gross and microscopic lesions, as well as other results obtained in this study on the pathogenicity in hamsters of dominant circulating Leptospira isolates in the Philippines, are serovar specific merits further investigation as we used only one strain representing each serovar. However, results from this study of the hamster model of leptospirosis are similar to previously reported manifestations of Well’s disease, the severe form of leptospirosis, in humans (Evangelista & Coburn, 2010; Arean, 1962). The findings observed in the experimental infection of hamsters in this study may explain the changes in recent reports regarding the pathogenicity of leptospires in humans, such as pulmonary haemorrhage, etc. This may also be a reflection of human leptospirosis, at least in the Philippines, as hamsters are known to be the best animal model for susceptibility to leptospirosis (Haake, 2006). As mentioned earlier, the four strains used in this study each represent the four dominant groups of leptospires that were previously isolated from the kidneys of rats in the Philippines (Villanueva et al., 2010). The PFGE results of our study showed similar fingerprint patterns among different human and rat isolates in the same country (unpublished data). Therefore, it is expected that Filipino leptospirosis patients may present with a severe form of the infection. Therefore, we stress the importance of formulating or strengthening preventive and control measures against this infectious disease, and measures towards the eradication of the reservoirs of infection (i.e. rats) should be established.

In summary, the results of this study showed the severity of infection among hamsters caused by the high virulence of the dominant circulating Leptospira serovars in the Philippines. Furthermore, the differences in pathogenicity seen amongst the strains belonging to L. interrogans (K64, K37 and K5) and that belonging to L. borgpetersenii (K6) may also be due to their different interactions with the host’s immune system, as also suggested in a previous study (Zuerner et al., 2012).

ACKNOWLEDGEMENTS

The authors are very grateful for the advice given by Dr Maria Ivy T. Clemente, Laboratory Director/Pathologist at the Lyndon B. Johnson Tropical Medical Center (American Samoa), especially on histopathological studies. This work was supported by grants received from the Special Coordination Funds on Science and Technology of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) and the Science and Technology Research Partnership for Sustainable Development (SATREPS) program of the Japan Science and Technology Agency (JST) and Japan International Cooperation Agency (JICA). The authors have no conflicts of interest to declare.

REFERENCES


