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Short communication

Rattus norvegicus as a model for persistent renal colonization by pathogenic *Leptospira interrogans*

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Abstract

Leptospirosis continues to be a disease with a poorly understood pathogenesis. The experimental rat model is amenable for the investigation of leptospiral dissemination, tropism, persistence of renal colonization and factors related to disease resistance. In this study, Wistar rats were infected intraperitoneally with virulent *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130. The detection of leptospires in tissue samples was based on culture, silver staining and immunofluorescence techniques. An inoculum of 10,000 leptospires induced colonization in 50% of rats and colonization persisted for the 4-month period of the study. Dissemination kinetics revealed that renal colonization took place 7–9 days after infection, with no underlying histopathology. The peak leptospiral load occurred on day 5 post-infection, followed by rapid clearance in all tissues except the kidneys, where dense leptospiral aggregates persisted in the renal tubules. We conclude that the experimental rat model is suitable for studies contributing towards the understanding of the mechanisms of colonization and resistance to severe disease in leptospirosis.

Keywords: Leptospirosis; *Leptospira*; Rats; Animal disease models

1. Introduction

Our knowledge of host-pathogen interactions has expanded with the use of experimental animal models of susceptibility and resistance to infectious disease. In the case of leptospirosis, a widespread zoonosis with a broad spectrum of clinical presentations and outcomes, experimental data is mainly limited to models of susceptibility in guinea pigs and hamsters (Faine et al., 1999). Pathogenic leptospires can infect diverse species of mammals and the *Rattus* genus has long been recognized as an asymptomatic chronic reservoir (Ido et al., 1917). The rat is also the most important transmission source in human infection in the urban environment (Bharti et al., 2003, Sarkar et al., 2002).

Early studies in rat models noted a marked natural resistance to lethal disease which could be overcome by administration of ethionine and cyclophosphamide (Bertok et al., 1964; Thiermann, 1980). The basis of species selectivity is associated with increased persistence in the renal tissue of the host animal. It was reported that *Leptospira* serogroups Grippotyphosa and Icterohaemorrhagiae were able to establish renal colonization in rats but that only the latter persisted for >7 months (Thiermann, 1981). Recently, the *R. norvegicus* model was used to contrast the chronic, asymptomatic, leptospiral infection with the acute, lethal, infection seen in the guinea pig model (Nally et al., 2005a). Mouse models have been used to investigate the role of specific genes in susceptibility (Nally et al., 2005b; Viriyakosol et al., 2006) however, mice are not an ideal model of resistance to acute disease as variations in strain, inoculum size and mouse age are known to affect outcomes (Faine, 1962).

Rats are an ideal model to study renal colonization since this process does not result in death or any notable histopathology. To date the dissemination kinetics of leptospires in rats has not

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been compared to what is known for susceptible hosts. In this report, we describe the establishment of a model of renal colonization in Wistar rats and report the influence of inoculum, time of persistence and dissemination kinetics on selected tissues.

2. Materials and methods

2.1. Strains

Leptospires were cultivated in liquid Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (Difco) at 29 °C and counted in a Petroff–Hausser counting chamber (Fisher Scientific) as described previously (Faine et al., 1999). A virulent clinical isolate from Brazil (Ko et al., 1999), *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, was used in all experiments. This strain was passed four times in hamsters and stored at −70 °C. Before use, the aliquots were thawed and passed in liquid medium seven times prior to use as a low-passage isolate in the infection experiments.

2.2. Rat model of leptospirosis

Four to 5-week-old Wistar rats (*R. norvegicus*) (Fiocruz, Rio de Janeiro, Brazil) inoculated intraperitoneally with various doses of leptospires in 1 ml EMJH medium, were sacrificed at intervals ranging from 1 day to 4 months after infection. Control animals were injected intraperitoneally with 1 ml of sterile EMJH medium. Necropsies were performed immediately upon sacrifice and kidney, spleen, lung and liver samples were stored at −70 °C in mounting medium. The remaining tissue samples were fixed in 4% formalin, embedded in paraffin and cut into 4–5 μm sections for conventional histology. Paraffin embedded sections were stained using the Warthin–Starry technique as previously described (Faine, 1965). The Ethical Committee of the Oswaldo Cruz Foundation approved all animal experimental protocols used in this study.

2.3. Re-isolation of leptospires from renal tissue

Renal tissue samples were homogenized in 10 ml of EMJH liquid medium for 10 min, followed by inoculation into 5 ml EMJH liquid medium and incubation at 29 °C. Urine was collected by aseptic puncture of the bladder during necropsy and 10 μl of urine was used to inoculate 5 ml EMJH liquid medium. The cultures were examined regularly for growth by darkfield microscopy for up to 26 weeks.

2.4. Immunofluorescence

Frozen sections (4–5 μm) were thawed on glass slides and incubated with a primary polyclonal antibody (prepared by immunizing New Zealand White-rabbits with a whole-cell preparation of *L. interrogans* serovar Icterohaemorrhagiae strain RGA) at a dilution of 1:800. After three washes in PBS, the sections were incubated with goat anti-rabbit IgG-FITC conjugate (Jackson Immunoresearch) at 1:800, washed three times in PBS and mounted in glycerol and antifade (*n*-phenylenediamine). Frozen tissue sections from non-infected rats were used as negative controls. Leptospires were counted in ten fields of view at ×400 magnification to determine the leptospiral load as described previously (Merien et al., 1998). The definition of established renal colonization was the presence of leptospiral aggregation limited to the tubular lumens of renal tissue.

2.5. Scanning electron microscopy of rat tissue samples

Tissue samples from animals with proven renal colonization (by renal culture and Warthin–Starry silver impregnation) were processed for scanning electron microscopy (SEM). Samples were prepared as 1–2 mm sections and immersed for a minimum of 1 h in 2.5% glutaraldehyde, 0.1 M sodium cacodilate (pH 7.2). Tissues were washed in 0.1 M sodium cacodilate (3×, 10 min) and post-fixed in 1% osmium tetroxide. Sections were washed, dehydrated in graded acetone followed by critical point and gold metallization and observed with a scanning electron microscope (Jeol 5310).

3. Results

Two experiments were performed to determine the minimum inoculum required to establish renal colonization 28 days after infection. Colonization was evaluated by Warthin–Starry silver impregnation and by culture isolation. In the two groups inoculated with 10^8 and 10^6 leptospires, all rats (6/6) were culture positive for renal infection, 3/6 rats inoculated with 10^4 leptospires were positive for renal culture while none (0/6) of the rats inoculated with 10^2 leptospires or those in the control group (0/2) showed evidence of renal colonization. The 50% colonization dose was calculated using the Reed and Muench method for calculation of 50% endpoints (Reed and Muench, 1938) and was determined to be 10^4 leptospires. A consistent outcome in all experiments was the resistance of the Wistar rats to acute lethal infection at all levels of inoculum and a lack of clinical symptoms of disease, the rats maintained their usual appetite, activity and posture.

In order to investigate the establishment and persistence of the carrier state, groups of rats, infected with 10^8 leptospires, were sacrificed at weekly intervals up to 1 month and then monthly up to 4 months. Based on the results from renal tissue culture, silver staining and immunofluorescence, renal colonization was established as rapidly as 1 week post-infection and persisted for up to 4 months (Table 1). SEM of kidney sections collected 28 days after infection confirmed the high density of leptospires in the tubular lumens of chronically infected rats (Fig. 1). Silver staining performed on other organs including liver, spleen, heart and lungs, was negative for the presence of leptospires.

The dissemination kinetics of leptospires was studied in rats (two experiments with three female rats per group) infected by peritoneal injection with 10^8 leptospires at 1, 3, 5, 7 and 9 days post-infection (Fig. 2). The peak leptospiral load in all tissues, based on silver staining and immunofluorescence, was observed on day 5 (Fig. 3). The liver had the highest leptospiral load (9.3 leptospires per ×400 field of view), however, leptospires

Table 1
Renal colonization of *R. norvegicus* by *L. interrogans* Copenhageni Fiocruz L1-130

Time post-infection	Renal colonization/total (%)		
	Renal/urine culture	Warthin–Starry	Immunofluorescence
1 Week	7/8[a] (88)	7/8 (88)	8/8 (100)
2 Weeks	8/8 (100)	8/8 (100)	8/8 (100)
3 Weeks	8/8 (100)	8/8 (100)	8/8 (100)
4 Weeks	19/21[a] (90)	19/21 (90)	8/8 (100)
2 Months	3/7[a] (43)	7/7 (100)	ND
3 Months	5/7 (71)	7/7 (100)	ND
4 Months	6/7 (86)	7/7 (100)	ND

[a] One non-positive culture due to contamination.

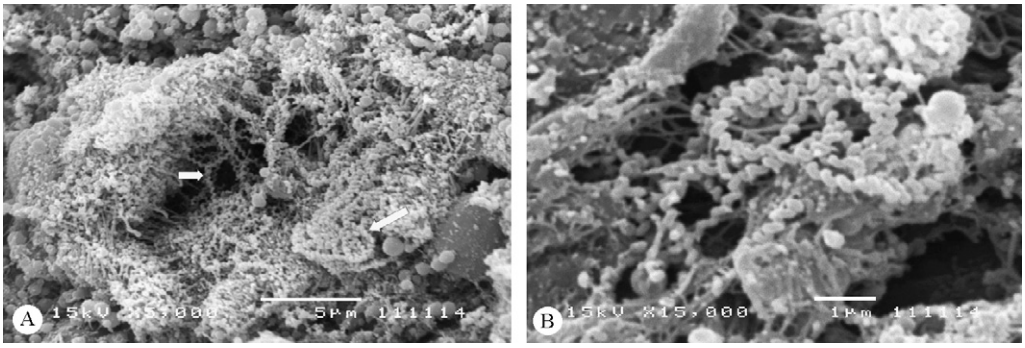

Fig. 1. Scanning electron microscopy of kidney tissue from a 4-week-old female rat 28 days after infection with 10^8 leptospires. The lumen of a renal tubule is shown (centre of image), containing a high density of leptospires with typical spiral morphology (A, ×5000 magnification). The short arrow indicates a leptospire within the tubular lumen. The long arrow indicates a leptospire attached to the inner tubular surface that was exposed by tissue fracture. The area outside the fractured tubule shows surrounding host tissue surfaces with no detectable leptospires and exhibiting small round bodies (blebs) indicating non-specific lesions of adjacent cells. An isolated leptospire is shown at the inner tubular surface (B, ×15,000 magnification).

were not detectable in any tissues except the kidneys after 7 days. Between days 7 and 9, intact individual and aggregated leptospires were observed in the kidneys by silver staining and immunofluorescence, indicating established renal colonization (Fig. 3). Leptospires were rarely observed in tissue samples from the lungs or eyes, on average less than one organism per field of view (×400). Light microscopy evaluation of kidney, lung, liver, heart, brain, skeletal muscle (diaphragm), eye and skin tissue samples revealed that the early dissemination process was not associated with any detectable lesions. The counting of leptospires in spleen tissue was complicated by the intense granular pattern of staining observed, possibly due to the presence of dead leptospires (data not shown). However, the majority (75%) of spleen sections, harvested between 7 days and 3 weeks after infection, exhibited signs of congestion in the chronic phase of infection.

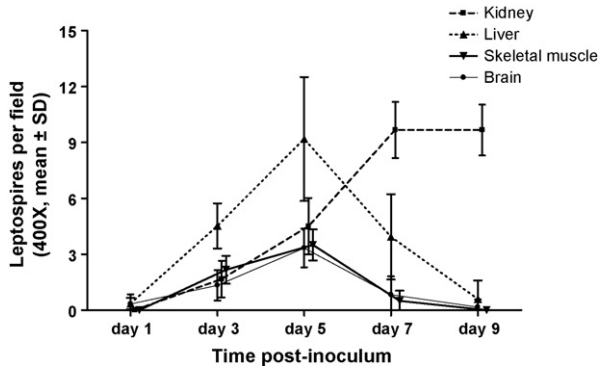

Fig. 2. Dissemination kinetics of pathogenic leptospires in Wistar rats. Results are expressed as an average ± standard deviation. The horizontal line represents the identified pattern of renal colonization with tubular aggregates.

4. Discussion

The results reported in this study confirm the resistance of *R. norvegicus* to severe acute leptospirosis. The establishment of an asymptomatic persistent renal carrier state was reproducible under experimental conditions, as previously reported (Nally et al., 2005a; Thiermann, 1981). The 50% colonization dose in rats was determined to be 10^4 leptospires in contrast to the low LD_{50} (<50 leptospires) observed in the Golden Syrian hamster model with the same infecting strain (Silva et al., 2007). Higher inoculum doses induced colonization in all rats and it is possible that high doses are required for transmission between rats. Rats live in colonies in close proximity to each other and in an environment highly contaminated by their urine. Although our understanding of how leptospires are transmitted between rats is limited, there is indirect evidence available from studies in which rat infestations were associated with higher rate of renal carriage (Faine et al., 1999). Concentrations of leptospires in urine are believed to be high, reaching 10^7 organisms/ml after

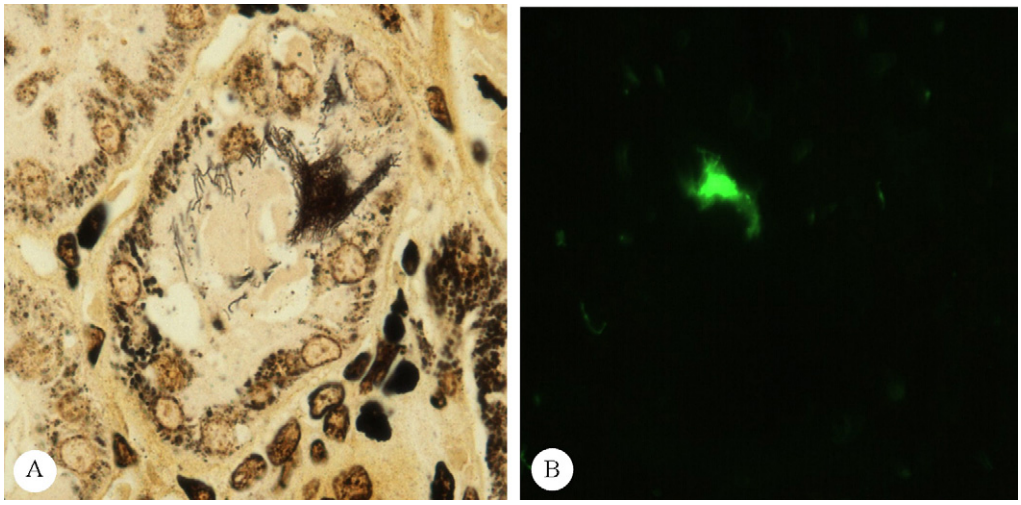

Fig. 3. Density of pathogenic leptospires by Warthin–Starry silver staining and immunofluorescence. (A) Infected kidney at day 9 showing leptospires detected by silver staining (×2000) and (B) infected kidney at day 9 visualized by indirect immunofluorescence showing intact individual leptospires and aggregates (×400).

experimental infection (Nally et al., 2005a) and therefore exposure to high doses in natural settings is probably the rule rather than the exception.

In the present study, chronically infected rats were studied for up to 4 months and all were positive by culture, silver staining or immunofluorescence. In a previous report, rats infected with a serogroup Icterohaemorrhagiae strain maintained a renal carrier state for 220 days compared to 40 days for a serogroup Grippotyphosa strain (Thiermann, 1981). Younger rats were reported to excrete serovar Copenhageni strain for only 72 days (Nally et al., 2005a). The variations in urinary shedding for the Icterohaemorrhagiae strains maybe due to the age of the animals used or to the different enumeration methods used (culture versus dark-field microscopy, respectively). In this study, the detection of leptospires *in situ* by a combination of three different methodologies (culture, silver staining and immunofluorescence) indicates that renal colonization is stable and long lasting. Immunofluorescence was the most sensitive method used in this study and was able to detect intact leptospires with a typical morphology, potentially indicating the number of viable leptospires. The major drawback was that chronic colonization of renal tubules could not be quantified due to leptospiral auto-aggregation. The *Leptospira* strain used in this study does not grow on solid agar, therefore viable counts to determine leptospiral load were not performed. Aggregation would also be a limitation for alternative quantitative methods such as endpoint dilution into culture medium. Potential alternatives for quantifiable detection include DNA amplification by real-time PCR, although this does not discriminate between viable and dead organisms (Palaniappan et al., 2005; Sunbul et al., 2001).

To confirm that the rat model is a viable one for the study of pathogenesis, we investigated whether the dissemination of leptospires in a resistant host shares similarities with the process seen in susceptible models for acute clinical infection. The results of this study suggest that in Wistar rats the leptospiraemic phase leads to rapid dissemination, followed by the accumulation of leptospires in the lumen of proximal tubules and the clearance of leptospires from all other tissues 9 days after infection. Similar behaviour has been reported in other experimental models including monkeys, mice, guinea pigs, hamsters, dogs and skunks (Alves et al., 1991; Branger et al., 2005; Faine, 1957; Marshall, 1976; Miller et al., 1974; Palmer et al., 1987; Tabel and Karstad, 1967). The dissemination process in the first week occurred without detectable pathology by light microscopy however, as we did not evaluate renal function or tubular transport, renal dysfunction in rats cannot be ruled out. In guinea pigs, densities of ≥100 leptospires per field of view (×400) were detected in the liver and between 10 and 100 bacteria in spleen, heart and lung tissues 4 days after infection (Merien et al., 1998). In our model, lower *Leptospira* densities were observed, in agreement with the *Leptospira* strain-dependant host adaptation and infection reported by Nally and co-workers (Nally et al., 2005a). Leptospires were rarely detected in lung tissue in our rat model, in contrast to studies with non-human primate and hamster models of acute leptospirosis (Miller et al., 1974; Pereira et al., 2005). This suggests that the rapid and widespread dissemination of leptospires is followed by the selective survival and proliferation of leptospires in the kidneys of rats. In addition, the high number of leptospires observed in renal tissues as early as day 7 may permit the recovery of sufficient leptospires for use in gene expression analysis.

The intraperitoneal route of infection may overestimate the pathogen load in tissues during dissemination and may create a bias for shorter dissemination times. How this observation reflects the conditions of natural infection or how environmental factors may influence the host-pathogen interaction is unknown. A previous report used a similar high dose (10^8) of leptospires, by subcutaneous injection, in the study of dissemination kinetics in the guinea pig model (Merien et al., 1998). The only study comparing infection outcome in guinea pig and rats used a high intraperitoneal inoculum (10^7) leptospires in Sprague–Dawley rats. Nally et al. suggested that changes in the expression of lipopolysaccharide O antigen may be involved in adaptation to host tissues, high expression was associated with chronic infec-

tion, but cause or effect was not established (Nally et al., 2005a). All other experimental infections in rats reported to date have used the intraperitoneal route (Bertok et al., 1964; Thiermann, 1980; Thiermann, 1981), including one study of long-term renal carriage where the lowest inoculum used was 10^7 leptospires (Thiermann, 1981). To our knowledge, the present study is the first to establish a colonization dose 50% for a reference strain with known virulence in a model of disease susceptibility. Natural routes of infection such as conjunctival inoculation need to be evaluated to determine if a bias does in fact occur in animals infected by the intraperitoneal route. We selected the intraperitoneal route in order to make the infection conditions analogous to those used in the hamster model (Silva et al., 2007).

In summary, a model of asymptomatic persistent renal colonization was established in Wistar rats using a highly virulent *L. interrogans* strain. The dissemination kinetics were similar to that seen in other mammalian hosts and were not associated with any underlying histopathology. The observation that leptospires initially disseminate extensively throughout the host, prior to clearance from all tissues except the kidneys, suggests that the kidneys are immune privileged sites and that this is not due to tissue tropism. Since the rat is the most important reservoir host for human infection, studies characterizing this animal model will be useful for future research on eradication strategies. The rat model has many potential applications in the study of leptospirosis pathogenesis, such as the factors involved during the infection and colonization process and in gene expression studies.

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