

Real-Time PCR Quantitation of *Leptospira* Tissue Burden in a Hamster Model

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Introduction

Leptospirosis is among the most common zoonotic diseases worldwide, affecting millions of humans and animals each year in both industrialized and developing countries, with fatality rates of up to 25% (Bharti et al. 2003, WHO 1999). Leptospirosis is caused by bacterial spirochetes of the genus *Leptospira*, a fastidious, slow-growing organism. The study of leptospiral pathogenesis in small animal models is hampered by difficulty quantitating the burden of infection because, although *Leptospira* grows efficiently in specialized liquid culture media, it grows poorly as colonies on solid media. We show here a real-time quantitative PCR (qPCR) assay that can quantitate *Leptospira* tissue burden in a hamster model of infection.

Pathogenic as well as nonpathogenic species of *Leptospira* can be found in water and soil. The life cycle of *Leptospira* differs from that of most bacteria, which typically replicate in the bloodstream and increase in number during the course of infection. In leptospiral infection, however, the leptospiremia phase lasts 5–7 days, after which the organism leaves the bloodstream and enters tissues, ultimately colonizing the proximal renal tubules. *Leptospira* can persist in the kidney for weeks or months and is shed into the urine and the environment.

Killed whole-cell vaccines to prevent human leptospirosis have been tested in human populations, most notably in Cuba, with unclear clinical efficacy and unacceptable side-effect

profiles (Martinez Sanchez et al. 2000). In addition to being an important human disease, leptospirosis has substantial significance in veterinary medicine, since infection can result in reproductive failure and other illnesses in horses, cattle, pigs, and dogs. Vaccines to prevent canine leptospirosis are widely used, and vaccines to prevent leptospirosis in cattle and other livestock are being developed. New vaccines must be continually developed to include new pathogenic serovars as they are discovered (Boutillier et al. 2003).

Hamsters and guinea pigs commonly serve as animal models for *Leptospira* vaccine development (Haake et al. 1999). Vaccine efficacy is evaluated based on protection against death or severe illness of the vaccinated animals following pathogen challenge. The utility of these small-animal models, however, is hampered when the pathogens do not lend themselves to traditional quantitation of blood or tissue burden of infection by quantitative colony counts. This is the case for *Leptospira* due to its slow growth on solid media, yet a critical threshold of leptospiremia may be significant in predicting clinical symptoms (Truccolo et al. 2001). Therefore, it is important to be able to measure the leptospiral organism burden in small animal models of leptospirosis.

In the experiments described here, we present a real-time PCR assay for quantitation of *Leptospira* in a hamster model and show that it can be used to measure bacterial burden in tissues. Speed, quantitative information, and ability to examine vaccine efficacy without animal sacrifice or severe morbidity are important advantages of this assay. This approach will be useful to experimental vaccine research and pathogenesis studies of leptospirosis in small-animal models.

Methods

Leptospira Infection

For these experiments, outbred female Syrian Golden hamsters obtained from Charles River Laboratories (Wilmington, MA) were used. All animal experiments described here were approved by the University of California, San Diego Institutional Animal Care and Use Committee and were performed in Association for Assessment and Accreditation of Laboratory Animal Care-approved Biosafety Level 2 animal facilities with approved biological safety procedures.

Three leptospiral strains isolated recently from patients in Iquitos, Peru were used for infection. These isolates, HAI0188, HAI0156, and VAR010, were associated with leptospirosis of varying severity. They were identified by a combination of group-specific serotyping, 16S ribosomal gene sequencing, and pulsed field electrophoresis gel fingerprinting (data not shown), and were maintained in liquid 1x PLM-5 leptospiral culture medium (Serologicals Corp., Norcross, GA). Organisms were quantitated using a Petroff-Hauser counting chamber under dark-field microscopy.

Groups of two hamsters were inoculated intraperitoneally with 10^8 organisms for each *Leptospira* strain; one animal was injected with 1x PLM-5 and used as a negative control. The animals were monitored daily. On day 3 following infection, one member of each group was sacrificed and the organs (lung, liver, kidney, and spleen) were removed aseptically to determine the bacterial load by real-time qPCR and for histological staining. The remaining animal in each group was sacrificed when moribund or at death, and the organs were harvested and processed similarly. Samples for PCR were stored in 70% ethanol at -80°C until needed. For histology, tissue sections were mounted in disposable cassettes (ProSciTech, Kirwan, Australia) and fixed in formalin overnight.

DNA Preparation and Real-Time qPCR

Total DNA for real-time qPCR was prepared from accurately weighed tissue samples (Table 1) using the DNeasy tissue kit (QIAGEN, USA) according to the manufacturer's directions. Real-time qPCR was performed using a previously described primer pair and probe (Smythe et al. 2002) labeled with the fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5' end, and the fluorescent quencher TAMRA (6-carboxy-tetramethylrhodamine) at the 3' end. The PCR primers, Lepto F (5'-CCCGCGTCCGATTAG-3') and Lepto R (5'-TCCATTGTGGCCGRA/GACAC-3'), allow amplification of the region between the positions 171 and 258 of the *rrs* (16S) gene, with an expected product size of 87 bp. The FAM-TAMRA labeled probe [5'-CTCACCAAGGCGAC-GATCGGTAGC-3'] spans the region from position 205 to 228. Reaction mixes were prepared using the Platinum quantitative PCR supermix-UDG kit (Invitrogen, Carlsbad, CA) with final

primer and probe concentrations of 600 nM and 100 nM, respectively, and 5 μl DNA extract (see Table 2 for details). Reactions were performed in triplicate. Amplification and fluorescent monitoring were performed on the DNA Engine Opticon[®] 2 thermal cycler using the following protocol:

1. Incubate 2 min at 50.0°C .
2. Incubate 2 min at 95.0°C .
3. Incubate 30 sec at 94.0°C .
4. Incubate 1 min at 50.0°C .
5. Read plate.
6. Repeat steps 2–5 for 44 more cycles.

To generate a standard curve, 13 mg of uninfected hamster kidney was spiked with 10^8 leptospires, extracted as described above, and used to prepare a 10-fold dilution series for real-time qPCR (Figure 1). The tissue burden of *Leptospira* for each sample was quantitated by interpolating threshold cycle (C_T) values against the standard curve. Samples with a C_T value >40 were considered negative.

Table 1. Weight of tissue specimens used for real-time qPCR experiments.

Isolate	Tissue	Weight (mg)	
		Hamster 1	Hamster 2
HAI188	Lung	13.1	19.6
	Liver	23.0	14.8
	Kidney	11.0	22.4
HAI156	Lung	5.2	21.4
	Liver	12.3	25.3
	Kidney	14.4	21.4
VAR010	Lung	9.0	6.0
	Liver	14.7	5.5
	Kidney	ND*	12.4

* ND, not determined.

Table 2. Real-time qPCR reaction components.

Component	Stock Concentration (μM)	Volume (μl)	Final Concentration (nM)
DNA template		5.0	Unknown
Forward primer	100	0.3	600
Reverse primer	100	0.3	600
Probe	50	0.1	100
Platinum quantitative PCR supermix	2x	25.0	1x
Water		19.3	
Total		50.0	

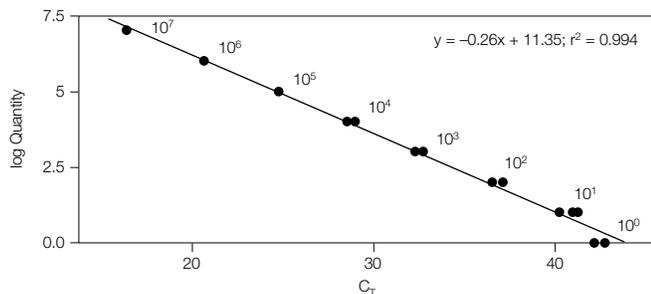


Fig. 1. Representative standard curve. A 10-fold dilution series of pathogen-free hamster kidney spiked with 10^8 leptospire was analyzed by real-time qPCR. Number of leptospire is shown next to data points.

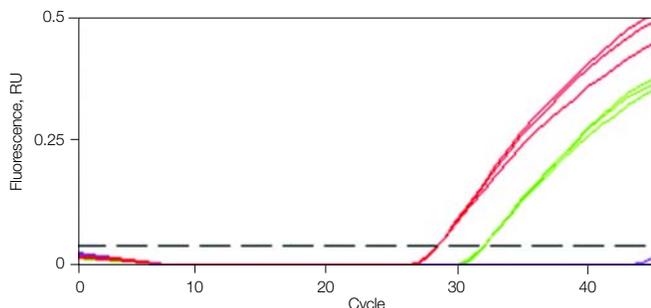


Fig. 2. Representative real-time qPCR results for leptospire detection in hamster kidneys after infection with the HAI188 isolate of *Leptospira*. Results shown are for tissue samples acquired 3 days postinfection (—) and at death 9 days postinfection (—). Results from kidney of an uninfected control animal injected with buffer only are also shown (—). Reactions were performed in triplicate.

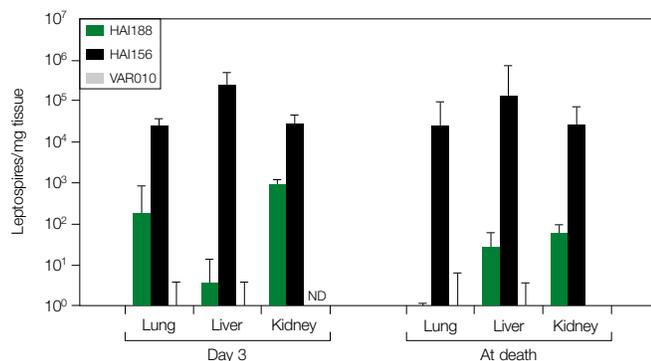


Fig. 3. Quantitation by real-time qPCR of tissue levels of three different *Leptospira* isolates in infected hamsters. Three samples from each tissue of each infected animal were analyzed. Values were interpolated by comparing the C_t value to the standard curve (Figure 1) and are reported as the mean number of *Leptospira* per milligram of tissue. Standard deviations are indicated by error bars. (ND, not determined.)

Results

We have developed a methodology that would allow us to determine the pathogenicity of leptospiral isolates in a small-animal model. Using fresh isolates obtained from humans with acute leptospirosis, groups of two hamsters each were inoculated with 10^8 *Leptospira* of three serovars: Icterohaemorrhagiae, which typically causes severe infection in humans; Canicola, which may or may not cause severe infection; and Varillal, a new serovar that is currently being characterized. The leptospiral isolates used for infection, HAI188, HAI156, and VAR010, correspond to the respective serovars, Icterohaemorrhagiae, Canicola, and Varillal. One animal from each group was sacrificed 3 days after infection and the other when moribund or at death to obtain lung, liver, and kidney samples.

Differences in the severity of disease were observed following infection and upon necropsy. After the first animal from each group was sacrificed 3 days after infection, the remaining hamster that had been injected with VAR010 had no observable clinical manifestations of illness and was sacrificed 15 days postinfection. At necropsy, there were no gross abnormalities. The hamster injected with HAI188 died 9 days after infection. At necropsy, the organs were grossly enlarged, particularly the spleen and kidneys. There was no evidence of jaundice, but focal hemorrhages in the lung were present. The hamster injected with HAI156 was moribund 5 days after infection and was sacrificed. At necropsy, the animal showed no evidence of jaundice. The spleen was enlarged, but there was no evidence of organ hemorrhage. A representative result obtained using qPCR to detect *Leptospira* in the kidney of the HAI188-infected animals and an uninfected control is shown in Figure 2.

Analysis of lung, liver, and kidney from all of the experimental animals by real-time qPCR for *Leptospira* identified substantial quantitative differences in the ability of the three leptospiral isolates to infect the hamsters (Figure 3). Infection with strain HAI156 resulted in the highest tissue load of leptospire and the most rapid death. There was a uniformly high bacterial load across the organs tested. Strain HAI188 also was lethal, and caused focal pulmonary hemorrhages in the hamster, similar to the illness it caused in the patient from whom the isolate was obtained. Strain VAR010, which did not cause illness in hamsters, was not detectable by real-time qPCR in the organs tested (lung, liver, kidney) at 3 or even 15 days postinfection when the animal was sacrificed. The negative control animal, injected with buffer alone, did not show any *Leptospira* load by real-time qPCR (data not shown).

Discussion

In this pilot study, we have demonstrated that a real-time qPCR assay can be used to quantitate leptospiral tissue burden in a hamster model of leptospirosis. We detected quantitative differences in the infectivity of different leptospiral isolates in hamsters. The data suggest that higher leptospiral burdens are associated with increased severity and more rapidly progressing disease, although additional experiments with larger numbers of animals are needed to confirm these observations.

Methods of quantitating pathogen load in small-animal models of infectious diseases differ among experimental systems. Straightforward methods can be used in some cases, such as colony counts on tissues for experimental *Salmonella typhimurium* infections, or examining blood smears for murine models of borreliosis, malaria, or African trypanosomiasis. For experimental infections such as leptospirosis, however, neither colony counts on tissues nor quantitative analysis of leptospiremia by microscopic examination are useful. *Leptospira* is too fastidious to grow quantitatively on solid media, and the number of leptospirems in the blood is too low for sensitive and accurate quantitation by microscopy. Others have successfully used PCR-based methods to quantitate leptospiremia in humans, showing that a critical threshold of $\sim 10^4$ leptospirems per ml of blood correlates with a poor prognosis (Truccolo et al. 2001).

In summary, real-time qPCR holds the promise of substantial utility for the sensitive detection of *Leptospira* in animal models of leptospirosis. The development and validation of this methodology for assessing tissue burden will help determine optimal points of intervention with vaccines or other treatments. Although only tissue levels of *Leptospira* were examined here using real-time qPCR, this methodology could be extended to testing of blood or urine, using appropriate methods of DNA extraction at time points corresponding to leptospiremia (leptospirems in blood) or leptospiruria (leptospirems in the urine). This less invasive method for blood and urine analysis thus offers the possibility of quantitative, noninvasive monitoring of infection and response to treatment.

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