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Research Paper

Characterization of a virulent *Leptospira interrogans* strain isolated from an abandoned swimming pool

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Abstract

Pathogenic *Leptospira* spp. are the etiological agents of leptospirosis, an important disease of both humans and animals. In urban settings, *L. interrogans* serovars are the predominant cause of disease in humans. The purpose of this study was to characterize a novel *Leptospira* isolate recovered from an abandoned swimming pool. Molecular characterization through sequencing of the *rpoB* gene revealed 100% identity with *L. interrogans* and variable-number tandem-repeat (VNTR) analysis resulted in a banding pattern identical to *L. interrogans* serogroup Icterohaemorrhagiae, serovar Copenhageni or Icterohaemorrhagiae. The virulence of the strain was determined in a hamster model of lethal leptospirosis. The lethal dose 50% (LD_{50}) was calculated to be two leptospires in female hamsters and a histopathological examination of infected animals found typical lesions associated with severe leptospirosis, including renal epithelium degeneration, hepatic karyomegaly, liver-plate disarray and lymphocyte infiltration. This highly virulent strain is now available for use in further studies, especially evaluation of vaccine candidates.

Key words: *Leptospira*, Leptospirosis, Virulent, VNTR, *rpoB*.

Introduction

Leptospirosis is a disease caused by pathogenic spirochetes belonging to the *Leptospira* genus (Faine *et al.*, 1999). It has a worldwide distribution and affects both humans and other animals (Bharti *et al.*, 2003), with more than 500,000 cases a year (WHO, 1999), however, it is still a neglected disease (WHO, 2011). The pathogen is maintained in the environment by susceptible or reservoir hosts that shed the bacteria in their urine, contaminating soil and waterways (Levett, 2001). Humans, an accidental host, typically become infected by direct or indirect contact with the urine of infected animals, particularly during seasonal flooding associated with heavy rainfall (Ko *et al.*, 1999, Lau *et al.*, 2010). Although human leptospirosis is considered an occupational hazard in developed countries, it is now more common in urban slums or in subsistence farming communities in underdeveloped countries (Mcbride *et al.*, 2005, Ko *et al.*, 2009). A wide variety of animals serve as potential reservoir hosts. The relative importance of a given reservoir species depends on the area, population density, the type of housing and the occupational and leisure activities of the local residents (Reis *et al.*, 2008). Leptospires are considerably resilient and are the only spirochaetes that can survive outside the host (Faine *et al.*, 1999). Isolation and characterization of pathogenic leptospires is important for the development of new vaccines and for improving diagnosis.

This study reports the isolation of a virulent *Leptospira* strain from a sample of water collected from a swimming pool in Southern Brazil and its characterization

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by sequencing of the *rpoB* gene and variable-number tandem-repeat (VNTR) analysis.

Materials and Methods

Leptospira spp. isolation and culture conditions

A sample of water was collected from an abandoned swimming pool, which contained dead possums and rats, in the city of Pelotas, RS, Brazil. A few drops of this water were used to inoculate 5 mL Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium supplemented with *Leptospira* Enrichment EMJH (Difco, BD Diagnostics, Sparks, MD, USA) and the cultures were incubated at 30 °C. After seven days, the culture was centrifuged at 5,000 x *g* for 5 min, the supernatant was passed through a 0.22 μm filter (Millipore, Billerica, MA, USA) and the filtrate was used to inoculate another tube of EMJH liquid medium. After nine *in vitro* passages, uncontaminated spirochete cells could be observed by darkfield microscopy. The isolate was named Spool, and stored in liquid nitrogen.

Genomic DNA extraction

A 10 mL culture grown for 7 days in EMJH medium was inactivated in a water bath at 56 °C for 30 min, centrifuged at 13,000 x *g* for 5 min, and DNA was extracted using Illustra Bacterium GenomicPrep Mini Spin kit following the manufacturer's instructions (GE Healthcare, São Paulo, SP, Brazil). The extracted DNA was analyzed by agarose gel electrophoresis to evaluate its integrity and quality, and stored at - 20 °C.

Partial sequencing of the *rpoB* gene

The hyper-variable region between base pairs 1900 and 2500 of the *rpoB* gene was amplified with primers Lept 1900f (5'-CCTCATGGGTTCCAACATGCA) and Lept 2500r (5'-CGCATCCTCRAAGTTGTAWCCTT) as previously described (La Scola *et al.*, 2006). DNA sequencing was performed using a MegaBACE 500 DNA sequencer and Dynamic ET-terminator technology (GE Healthcare). The resulting sequence was submitted to basic local alignment search tool (BLAST) alignment (www.ncbi.nlm.nih.gov/BLAST) to identify the *Leptospira* spp. by alignment with *rpoB* sequences in GenBank.

VNTR analysis

Seven discriminatory primers (VNTR4, VNTR7, VNTR9, VNTR10, VNTR11, VNTR19 e VNTR23) were used to characterize the isolate as previously described (Majed *et al.*, 2005). The size of the amplified products was analyzed by agarose gel electrophoresis as described previously (Sambrook and Russell, 2000). Genomic DNA purified from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was used as a positive control.

Western blotting

For Western blotting, a whole-cell extract was separated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane Hybond ECL (GE Healthcare) as previously described (Sambrook and Russell, 2000). After blocking the membranes were incubated with the anti-LipL32 1D9 MAb at 1:500 dilution in PBS or anti-LigA and LigB polyclonal mono-specific mouse sera at 1:100 dilution in PBS. After three washes with PBS containing 0.05% (v/v) Tween 20 (PBS-T), the membranes were incubated with an anti-mouse IgG peroxidase conjugate diluted in PBS-T. The reaction was developed with 4-chloro-1-naphthol (Sigma) after five washes with PBS-T. The BenchMark Pre-Stained Protein Ladder (Invitrogen, São Paulo, SP, Brazil) was used as molecular weight marker.

Virulence testing and histopathology

The virulence of the isolate was confirmed using the hamster model of lethal leptospirosis. The animals were housed at the animal facility of the Federal University of Pelotas (UFPel) and maintained in accordance with the guidelines of the Ethics Committee in Animal Experimentation, UFPel throughout the study period. Leptospires were counted in a Petroff-Hauser counting chamber (Fisher Scientific, Pittsburgh, PA, USA) as previously described (Faine *et al.*, 1999). Two nine-week-old male hamsters were inoculated intraperitoneally with 10^8 leptospires. Clinical symptoms were monitored by daily observations during the study period and moribund animals were euthanized immediately. Post-mortem, the kidneys, lungs and liver were collected and stored in 10% formalin for histopathological examination after staining with haematoxylin and eosin. One of the kidneys from each animal was macerated and inoculated into EMJH liquid medium for re-isolation.

Determination of the LD_{50}

Forty golden Syrian hamsters (nine-week old, 20 males and 20 females) were infected intraperitoneally with 10 fold serial dilutions (10^4 to 10^0 leptospires) in a final volume of 1 mL PBS (see Table 1). Animals were monitored daily for clinical symptoms until 30 days post-infection, where upon all survivors were euthanized. The LD_{50} was calculated by the method as previously described (Reed and Muench, 1938).

Results

The presence of dead rats and a possum in a swimming pool that had not received any type of water treatment for more than one year prompted us to try to isolate leptospires from a sample of the water. Although the primary culture was contaminated with other bacteria, a few spirochetes could be observed under darkfield microscopy.

After passing the culture through a 0.22 µm filter and sub-culturing in liquid EMJH medium for nine passages, a pure culture was obtained.

After DNA extraction, PCR amplification and partial sequencing of the *rpoB*, BLAST alignment with sequences in GenBank reported 100% identity with the *rpoB* gene from *L. interrogans* (data not shown). To further characterize the isolate, VNTR analysis was performed using seven VNTR loci. Analysis of the electrophoresis profile of the amplified VNTR fragments revealed an identical pattern between the isolate and the *L. interrogans* serovar Copenhageni L1-130 strain (Figure 1).

To further confirm the pathogenic status of the isolate, expression of the LipL32, LigA and LigB proteins, which are unique to pathogenic *Leptospira* spp., was evaluated by Western blotting. Expression of all three of the antigens was observed (Figure 2), confirming that the isolate was a pathogenic *Leptospira* strain.

In a confirmatory test for the virulence of the isolate, all hamsters that were inoculated with 10^8 leptospires went on to develop lethal leptospirosis (data not shown). Typically, clinical symptoms and death were observed on the second to fourth day post-infection (pi). The symptoms included: dehydration, ruffled hair coat, decreased activity

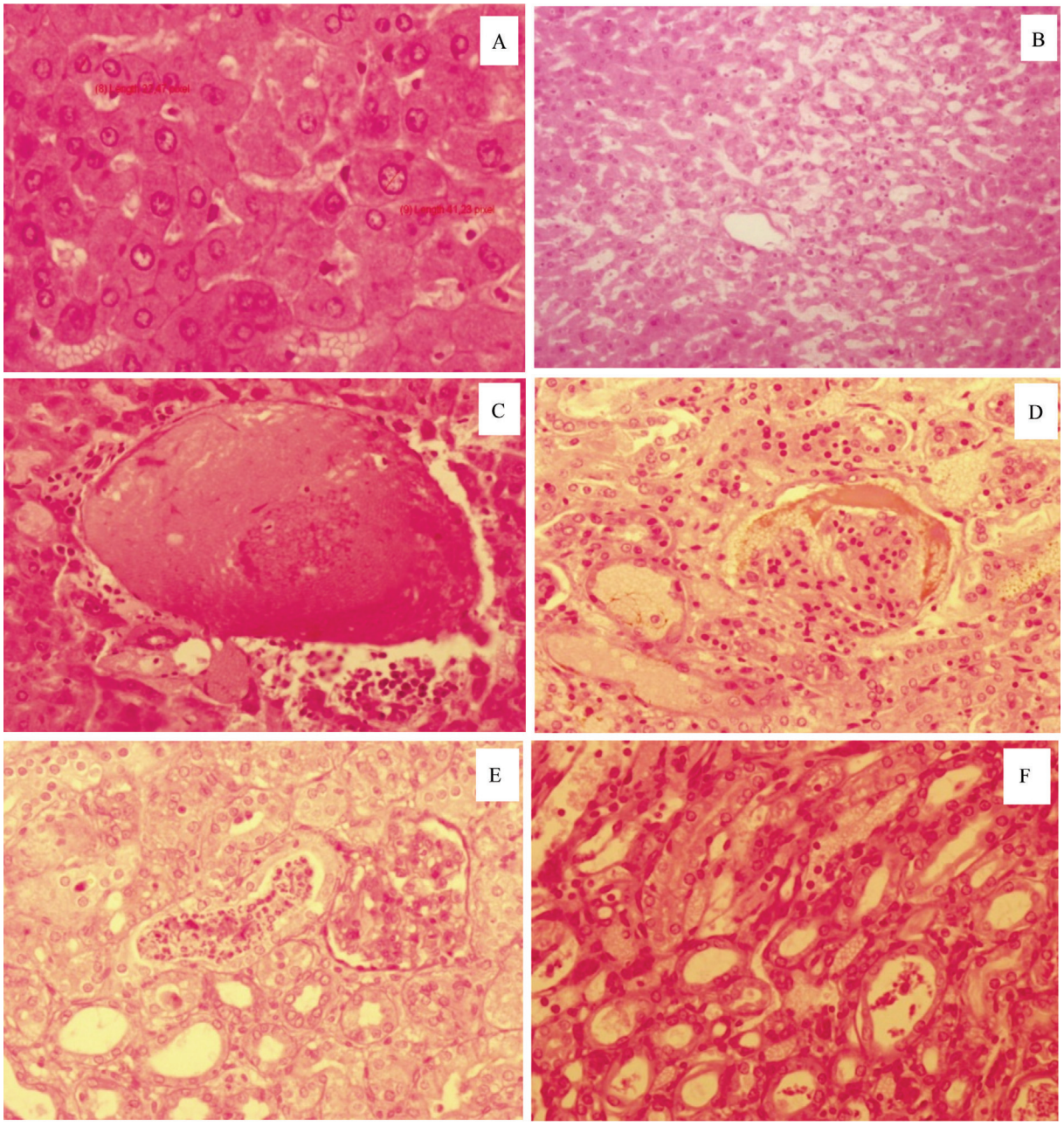

Figure 1 - Electrophoresis in 0.8% agarose gel. Columns 1 and 2 with molecular marker (1 kb DNA ladder, Invitrogen); (a) *L. interrogans* L1-130 used as positive control; (b) *L. interrogans* strain SPool.

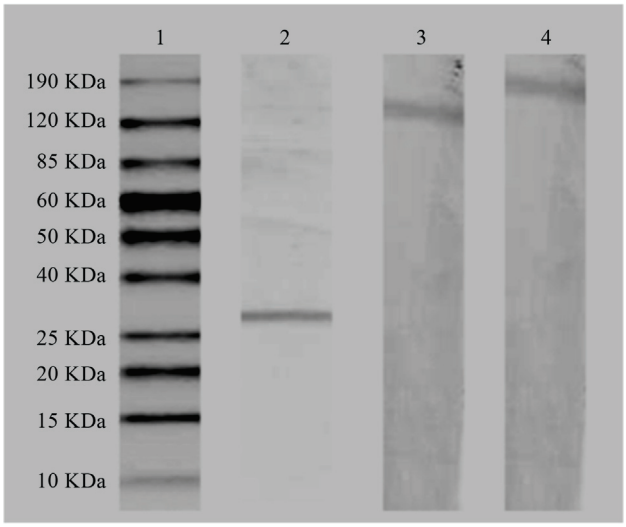

Figure 2 - Western blot of SPool isolate cell extract probed with different antibodies. Lane 1, BenchMark Pre-Stained ladder; lane 2, anti-LipL32; lane 3, anti-LigA; lane 4, anti-LigB.

and isolation. In addition, we observed severe external haemorrhaging, swelling and complete closure of the eyes. The histopathological results showed that the infected animals developed renal epithelium degeneration and hepatic cells with karyomegaly, diffuse loss of cohesion (liver-plate disarray), and lymphocyte infiltration (Figure 3).

In the experiments to determine the LD_{50}, death was observed to begin on day 11 pi, and continued to day 22 pi (Figure 4). None of the hamsters inoculated with 10 or more leptospires survived the infection. Inoculation with one leptospire resulted in death for 25% (1/4) of female and 50% (2/4) of male hamsters. The LD_{50} was calculated by the regression method of Reed and Muench, and was ~2 and ~1 leptospires for female and male hamsters, respectively.

Results and Discussion

The isolation and characterization of region-specific *Leptospira* isolates is essential towards understanding the

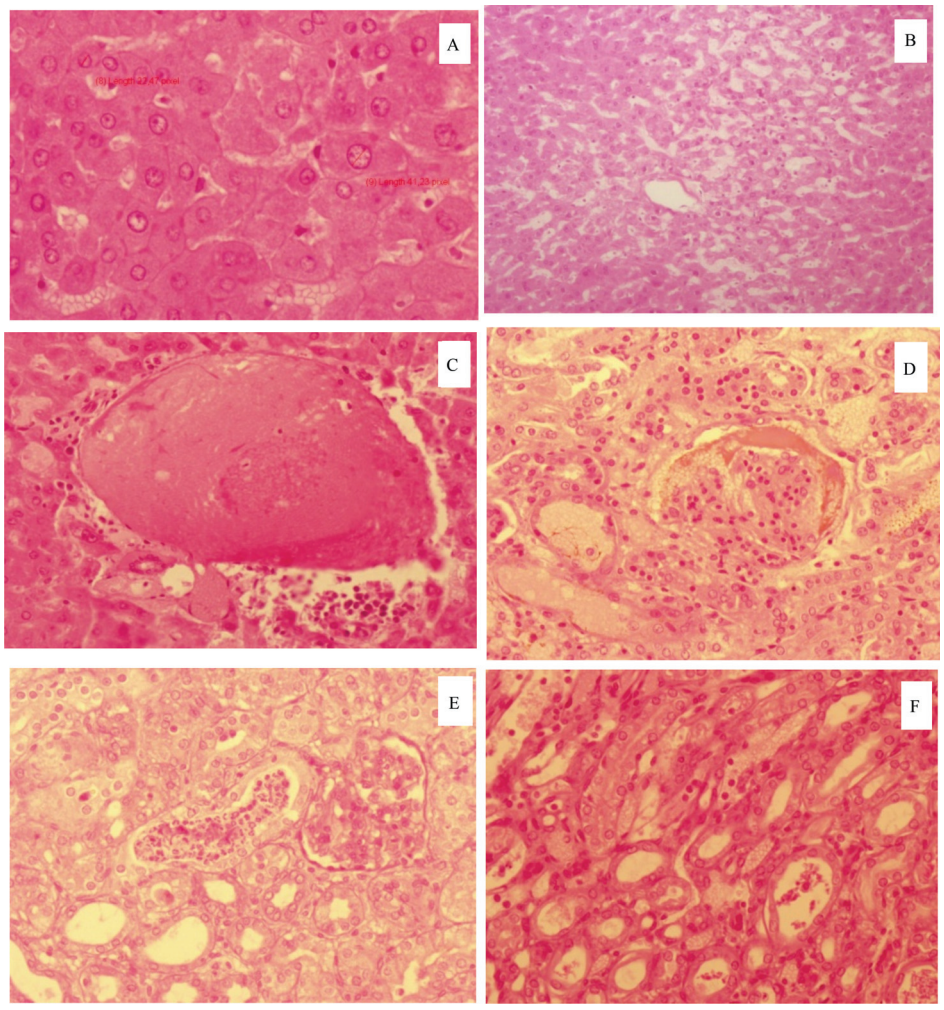

Figure 3 - Typical lesions of leptospirosis in a 9-week-old hamster that died four days after infection with *L. interrogans* strain SPool. (A) Hepatic cells with karyomegaly. (B) Diffuse loss of cohesion (liver-plate disarray) of liver cells. (C) Lymphocyte infiltration around the central vein. (D) Degenerate renal glomerulum with haemorrhagic oedema. (E) Leukocyte infiltration in the renal tissue. (F) Renal tissue with macrophages cells.

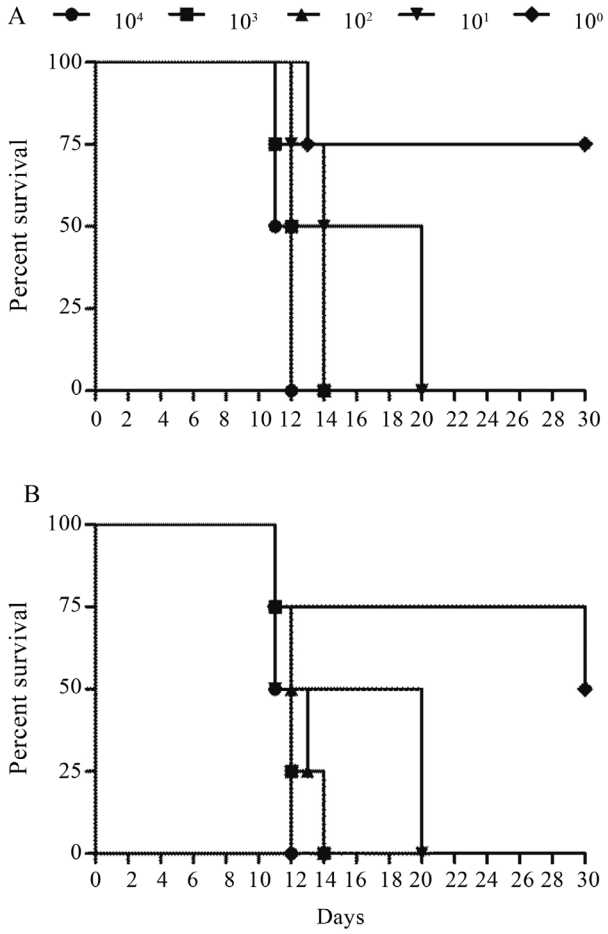

Figure 4 - Survival curves for calculation of the LD_{50} for the *L. interrogans* SPool isolate. Groups of female (A) and male (B) hamsters (n = 4) infected with 10^4, 10^3, 10^2, 10^1 and 10^0 leptospires.

epidemiology and improving diagnosis of leptospirosis (Bourhy *et al.*, 2010). In this study, we obtained an isolate of *Leptospira* from a sample of water collected from a swimming pool in the city of Pelotas, in the Southeast of Brazil. This swimming pool had not been cleaned or treated for more than one year and two rat and one possum carcasses were observed. After nine passages *in vitro* and filtering to remove contaminants, a pure culture was obtained, as determined by darkfield microscopy.

Molecular characterization by partial sequencing of the *rpoB* gene and VNTR analysis have become the standard molecular techniques used for identification to the species and serogroup level of *Leptospira* spp. (La Scola *et al.*, 2006, Cerqueira and Picardeau, 2009). VNTR analysis was carried out using the loci described by Majed *et al.* (2005). Based on the *rpoB* sequence alignments with sequences in GenBank, the isolate was identified as *L. interrogans*, belonging to the Icterohaemorrhagiae serogroup. The serovar was determined to be either Icterohaemorrhagiae or Copenhageni, however, it was not possible to discriminate between the two as these serovars have identical VNTR profiles (Figure 1).

The virulence of *Leptospira* spp. is known to be strain dependent (Faine *et al.*, 1999) and this was highlighted by the sequencing of two *L. borgpetersenii* serovar Hardjo strains, only one of which was virulent in hamsters (Bulach *et al.*, 2006). The hamster model reproduces the pathology observed in acute forms of human leptospirosis, as previously described (Haake, 2006, Dellagostin *et al.*, 2011). The typical pathology includes acute cell swelling and multifocal regeneration tubular foci and interstitial nephritis. Using the hamster model of leptospirosis the virulence of the SPool isolate was confirmed and the LD_{50} was calculated to be approximately two and one leptospires in the female and male models, respectively. Furthermore, various strains of *Leptospira* spp. can cause host-dependent symptoms and some of the symptoms are characteristic of a particular strain. In this study, the symptoms caused by the SPool strain included swelling of the eyelids resulting in complete closing of the eyes. This was an unusual finding and has not previously been reported.

To further characterize the SPool isolate the expression of several pathogenic-specific antigens was evaluated by indirect immunofluorescence and Western blotting. The expression of LipL32, the immunodominant surface-exposed antigen was confirmed using an anti-LipL32 MAb (Fernandes *et al.*, 2007). The *lig* genes encode virulence determinants involved in host-pathogen interactions and they have been used for diagnosis and as vaccine candidates (Dellagostin *et al.*, 2011). LigA and LigB expression was detected using specific polyclonal mouse sera.

Due to the severe side effects associated with the currently used whole-cell (bacterin) type leptospirosis vaccines there is a need for an alternative, recombinant vaccine for the prevention of leptospirosis (Dellagostin *et al.*, 2011). A more complete evaluation of the capacity of vaccine candidates to induce a cross-protective immune response requires a well characterized panel of virulent strains representing the *Leptospira* serovars of public health and veterinary importance (Silva *et al.*, 2008). With the isolation of the SPool strain we report on the availability of an additional strain for evaluation of vaccine candidates. Previous studies of vaccine candidates reported variable efficacies and this was suggested to be due to virulence of the challenge strain (Adler and Moctezuma, 2010). Weakly virulent strains requiring challenge doses of $\geq 10^6$ leptospires often resulted in survivors in the control groups. The LD_{50} of the SPool strain is approximately 2 leptospires, one of the lowest described to date.

In conclusion, we report the isolation and characterization of a pathogenic isolate of *L. interrogans* obtained from a swimming pool. This highly virulent strain will be used in experiments evaluating the protection afforded by novel recombinant vaccine candidates against leptospirosis currently being evaluated in our laboratories.

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