

DIAGNOSIS OF CANINE LEPTOSPIROSIS USING AN IMMUNOMAGNETIC SEPARATION-PCR METHOD

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ABSTRACT

Diagnosis of leptospirosis by PCR is hampered due to the presence of substances on biological fluids. Here, we report an immunomagnetic separation step prior to PCR which improved the detection of *Leptospira* spp. in blood and urine samples from dogs. It resulted in a significant improvement on sensitivity for diagnosis of canine leptospirosis.

Key words: Canine leptospirosis, IMS-PCR, LipL32.

Leptospirosis is a zoonosis of ubiquitous distribution, caused by infection with pathogenic *Leptospira* spp., which occurs in many animal species and humans (1). Dogs may be exposed to leptospires in the environment by contact with urine of an infected host, contaminated water or moist soil, where the bacteria may survive for several months (5, 12). In the last years, several assays have been proposed for confirmation of canine leptospirosis, including serology, polymerase chain reaction (PCR), fluorescent antibody testing of urine or tissue samples, or organism isolation (8).

The recommended laboratory diagnostic test, microscopic agglutination test (MAT), is based on detection of antibodies against *Leptospira* spp. in dog sera; however, pathogen-specific antibodies may remain in the blood stream for a long

period, even after recovering from disease. Besides serology, demonstration of leptospires by culture of blood, tissues or urine is definitive; it also identifies the infecting leptospire (7).

PCR assay is highly sensitive, but the presence of PCR inhibitory substances on biological fluids can prevent amplification, resulting in false negative (10). Recent studies report the immunomagnetic separation (IMS) technique prior to PCR assay as an approach to reduce the effect of inhibitory substances present in biological fluids and food samples (2, 3, 4, 6, 7, 9, 14, 15). For diagnosis of leptospirosis, the IMS-PCR approach was reported to detect *Leptospira* spp. in bovine urine (15) and in human biological fluids (6). In order to improve PCR sensitivity and specificity, we developed a novel IMS-PCR approach by using both magnetic beads in house coated

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with a monoclonal antibody (mAb) and specific PCR primers for pathogenic *Leptospira* spp. (6, 9). Blood and urine samples were obtained from dogs suspected of having leptospirosis (n=5) at the Veterinary Hospital/ Universidade Federal de Pelotas, Brazil. Blood samples were centrifuged at 2,000 x g for 5 min, the supernatant was collected, and one drop was observed under microscope to investigate the presence of spirochetes by darkfield microscopy (DFM) on an Olympus BX 51 microscope. Control samples were obtained from healthy dogs (n=5) negative by MAT. The MAT was performed according to Faine *et al.* (5), using reference strains of 19 different leptospiral serovars. Reciprocal agglutination titres of greater than or equal to 1:100 were considered positive reactions.

For PCR assay, blood sera and urine samples were centrifuged at 15,000 x g for 10 min. The cells were washed with sterile 0.01 M phosphate-buffered saline (PBS, pH 7.2) and the DNA was extracted by heating at 95 °C for 15 min in 50 µL of lysis solution (1:1 of 0.125% SDS and 0.05 M NaOH). In experiments in which lysis followed IMS, 20 µL of the lysis solution were directly added to immune separated products in microtubes and then heated at 95 °C for 15 min. Primers *lipL32* F: 5' CGCTTGTTGCTTCGGTGGT 3' and *lipL32* R: 5' CTCACCGATTTCGCCTGTTGGG 3' were used, resulting in a 264 bp amplicon of the *lipL32* coding region (9). Amplification was carried out in a Peltier Thermal Cycler PTC-100® (Bio-Rad) with 1 cycle at 94 °C for 5 min, 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by an extension for 7 min at 72 °C at the end of the final cycle. Aliquots were analyzed by electrophoresis in 1% agarose gel with ethidium bromide and visualized under UV transillumination. A specific PCR internal amplification control (IAC) was used in all assays, which consists of a DNA fragment containing 501 bp not related to *Leptospira* spp., flanked by target sequences of *lipL32* primers (6). The IAC concentration was estimated spectrophotometrically at 260 nm and the optimal concentration for use in the *lipL32* PCR was

determined by titration, and the lowest reproducible concentration was determined using decimal dilutions of IAC (50 to 0.05 pg) as template DNA in a PCR with *lipL32* primers. PCR and IAC-PCR were performed using a single pair of *lipL32* specific primers.

Determination of the minimal concentration of DNA and number of leptospiral cells required to result in amplification of the *lipL32* gene sequence by PCR was carried out according to Fernandes *et al.* (6). Briefly, genomic DNA from *L. interrogans* was diluted with sterile 10 mM Tris-HCl, 1 mM EDTA (TE, pH 8.0) to concentrations ranging from 20 to 1 pg/µL, and pellets from control samples artificially contaminated with *Leptospira interrogans* serovar Canicola strain Hond Utrecht IV at concentrations ranging from 10⁸ to 10⁰ cells per mL. Then, the pellets from each DNA concentration and bacterial dilution were washed with PBS and suspended in 50 µL of lysis buffer for DNA extraction. DNA extracted from saprophytic *L. biflexa* serovar patoc Patoc I or *E. coli* were used as negative controls. These experiments were repeated three times.

The IMS-PCR was performed with protein A-magnetic beads (Bangs Laboratories Inc, Fishers, IN, USA) adsorbed with a mAb against leptospira LipL32 protein according to the manufacturer's instructions. The mAb used in this work is specific to pathogenic leptospires (6). Briefly, 10 µL of mAb-coated beads were added to clinical and control samples. The immunocapture complex was washed three times, suspended in DNA extraction buffer, boiled and used on PCR assay. To perform the experiments, an immunomagnetic separator MPC-S (Invitrogen, CA, USA) was used.

All 5 dog serum samples were MAT positive with titers that varied from 100 to 3200. The antibodies most frequently found in the MAT recognized serovars Canicola CCZ463 (5/5), Canicola Hond Utrecht IV (4/5), Icterohaemorrhagiae (4/5), Copenhageni (4/5), Ballum (3/5) and Grippotyphosa (3/5). The highest agglutination titers corresponded to serovars Canicola CCZ463 and Canicola Hond Utrecht IV. No agglutination was

observed in sera from healthy dogs. Conventional PCR assay was able to detect 2 pg per 25 μ L of pure genomic *Leptospira* DNA and 10^3 cells per mL either in artificially contaminated canine serum or urine samples (data not shown). When IMS was applied on artificially contaminated samples prior to PCR the detection limit decreased to 10^2 cells mL $^{-1}$ (Fig. 1A). No amplification was observed after IMS-PCR performed with saprophytic strains or *E. coli*, or when non-sensitized beads were used for IMS with

pathogenic strains (data not shown). PCR performed without previous IMS treatment amplified *lipL32* gene sequence from four urine and one serum samples (data not shown). The IMS-PCR performed with canine leptospirosis clinical samples was able to amplify *lipL32* sequence gene in all urine and in two out of five serum samples tested. These results are demonstrated in Fig. 1B. The IMS-PCR approach enhanced the PCR method since the conventional PCR failed to detect one positive sample.

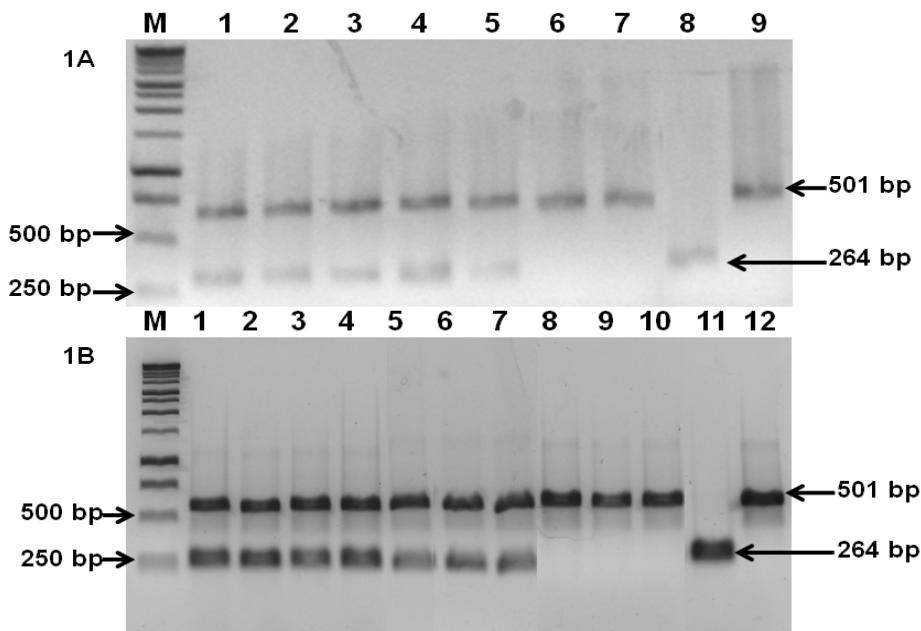


Figure 1. Agarose electrophoresis of IMS-PCR from artificially contaminated and clinical fluid samples in presence of 0.5 pg of IAC. Panel A, detection limit of *L. interrogans* serovar Canicola strain Hond Utrecht IV in artificially contaminated dog urine samples: M, 1 kb DNA Ladder; Lanes 1-7, 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , 10^0 leptospires per mL, respectively; lane 8, DNA only, lane 9, IAC only. Panel B, detection of *Leptospira* spp. amplified from dog clinical samples. M, 1kb DNA Ladder; lane 1-5 (urine) and 6-10 (blood), respectively. Lane 11 DNA only; Lane 12 IAC only.

Canine leptospirosis has a variable clinical presentation but, as a consensus, it results in leptospira renal scarring (5). The recommended diagnostic test has to be done by testing paired acute and convalescent sera to confirm the diagnosis, which is helpful in unvaccinated dogs, but hard to interpret in vaccinated ones (6). The antibodies detection methods are not useful before seven days after leptospira infection and the

standard method, MAT; require paired samples to detect seroconversion (5). For those reasons, antigen detection tests offer potential advantage over tests based on antibody detection both for early diagnosis and identification of renal carrier status (5). Therefore, research focused on highly sensitive and specific routine tests for leptospira detection in blood and urine samples led to development of several molecular methods for

diagnosis of leptospirosis (4, 6, 9, 14). However, the sensitivity and kinetics of PCR diagnostic tests may be dramatically reduced when applied directly to biological samples, such as urine and serum samples (11). The presence of inhibitor molecules and heterogeneous bacteria in the samples can affect PCR performance (13).

Here, we demonstrated that the IMS step can be useful for concentrating leptospires in clinical samples to allow detection by PCR and for reducing inhibitory substances which led to increase in sensitivity. Although the IMS-PCR was able to detect leptospires in only two out of five serum samples tested, high agglutination antibodies titers were found in those sera and no spirochetes were visualized under DFM suggesting absence of leptospiremia in those animals. The use of a sensitive leptospira detection method such as the IMS-PCR, may constitute an important tool for identification of leptospira renal scarring.

In conclusion, this study demonstrated that IMS using an extensively characterized mAb against LipL32, a surface exposed outer membrane protein present in all pathogenic leptospires, is efficient in capturing pathogenic leptospiral cells. In addition, the IMS coupled to PCR has the potential to improve sensitivity and specificity of a diagnostic test for leptospirosis. The same approach may be useful for detection of other pathogens.

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