Artículo original

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Comparison between real-time PCR, serology and culture in leptospirosis, from samples of wild animals trapped in Buenos Aires Province, Argentina

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Abtract
The definitive diagnostic test for leptospirosis is the recovery of leptospires from the culture of clinical samples. The micro- agglutination test (MAT) which has high sensitivity and specificity, is a reference standard test for serum diagnostic. The advent of molecular methods has facilitated the diagnosis of leptospirosis by PCR reaction. The aim of this study was to compare the results obtained by using bacteriology (culture), serology and molecular biology, from samples of wild animals. The 8.8% of positive samples was obtained by MAT (two *R. norvegicus* and *L. griseus* positives). Kidneys from all animals were cultured, and two isolate (2/34) of *L. interrogans* from *R. norvegicus* was obtained (6.9%). Serum sample was studied by real-time PCR and 17/34 was positive (50%). Isolation of leptospires from clinical samples is strong evidence to confirm the diagnosis in order to identify the serotypes circulating in a particular geographic region, and in turn be used as antigens in MAT. In real-time PCR from serum samples time, proved to have high sensitivity, being an important tool for the diagnosis of leptospirosis, especially in acute cases of disease, in which the other techniques often provide negative results.

Keywords: wild animal's leptospirosis, culture, microscopic agglutination test, real-time PCR.

Comparación entre PCR en tiempo real, serología y cultivo en leptospirosis, a partir de muestras de animales silvestres capturados en la provincia de Buenos Aires, Argentina

Resumen
La recuperación de leptospiras a partir del cultivo de muestras clínicas es el diagnóstico definitivo de la enfermedad. La prueba de micro aglutinación (MAT) que tiene una alta sensibilidad y especificidad, es la prueba de referencia. La llegada de los métodos moleculares ha facilitado el diagnóstico de la leptospirosis mediante técnicas de PCR. El objetivo del presente trabajo fue comparar los resultados de bacteriología (cultivo), serología y biología molecular, a partir de muestras de animales salvajes. Por medio MAT se detectó un 8.8% de positividad (dos *R. norvegicus* y un *L. griseus*). Se aislaron leptospiras en 2/34 cultivos de riñón (6.9%). Mediante PCR en tiempo real se detecta 17/34 animales positivos (50%). El aislamiento de leptospiras a partir de muestras clínicas es la evidencia para confirmar el diagnóstico, permitiendo identificar los serotipos que circulan en una región geográfica determinada, y a su vez ser utilizados como antígenos en MAT. La PCR en tiempo real a partir de muestras de suero, demostró tener una alta sensibilidad, siendo una herramienta importante para el diagnóstico de la leptospirosis en casos agudos de enfermedad, donde las otras técnicas a menudo proporcionan resultados negativos.

Palabras claves: leptospirosis en animales silvestres, cultivo, prueba de aglutinación microscópica, PCR en tiempo real.

Introduction

Leptospirosis has a worldwide distribution, and is an emerging infectious disease of humans. Wild animals, mainly the rodents, are considered the principal reservoirs of many serovars of leptospires[1-4]. The definitive diagnostic test of leptospirosis in these animals is the recovery of leptospires from clinical samples, either by culture; nevertheless, this technique is not sensible enough and requires qualified staff. Cultures should be incubated between 28 and 30 °C and observed weekly using a dark field microscopy; this bacterium grows slowly and cultures should be informed negative after a three-month minimum of observation[5]. In the first 7 to 10 days of disease, leptospires can be isolated from blood samples, while urine samples can be taken from the second week of the course of disease. However, the urine culture is not frequently used because the samples are frequently contaminated. Renal tissue extracts are used to attempt to isolate leptospires; being rodents (*Rattus sp*.) the wild animals more studied[1-4]. The microscopic

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agglutination test (MAT) is the reference standard test for serological diagnosis of leptospirosis because of its high sensitivity and specificity[6, 7]. The MAT detects agglutinating antibodies in serum, but it is a complex and difficult test, for realization and interpretation, reason why it needs staff with experience and maintenance of the lively cultures of the serovares used as antigens[8]. It is a very useful test in seroepidemiological investigations of serum samples from the general population, and may indicate circulating serogroups since residual antibodies from past infections tend to react with serogroup-specific antigens. The arrival of the molecular methods has facilitated the diagnosis of leptospirosis by PCR (polymerase chain reaction), demonstrating the presence of DNA of leptospires in clinical samples of serum, blood and urine, by means of the use of specific primers which amplify saprophytic and pathogenic leptospires[8-12]. PCR is a technique that allows the amplification of specific DNA sequences of leptospires from a DNA sample, being the products of amplification visualized in agarose gels under U.V. light. A conventional limitation of the diagnosis of the leptospirosis by PCR is the inability to identify serovars. In wild animals, studies by conventional PCR allowed getting high rates of detection of *Leptospira* DNA from renal tissue samples[13, 14]. PCR has also been used to distinguish pathogenic from non-pathogenic *Leptospira* species in clinical and environmental samples[10-12]. The objective of the present work was to compare the results between real-time PCR, serology and culture, from samples of wild animals trapped in Buenos Aires province, Argentina, and provides data on leptospirosis kinetics in reservoir animals.

Material and methods

Wild animals were captured with tomahawk traps in rural and peri-urban area of Azul, Lamadrid and Tandil, Buenos. Traps were baited with animal fat and checked every morning for three consecutive days. Captured animals were euthanized according to Animal Welfare Committee of the Veterinary Sciences Faculty, National University of the Center of Buenos Aires province, Argentina (Number interne dispatch: 13). None of the animals trapped were at risk of extinction. Animals were classified as adults or juveniles based on genital development and body size. Necropsies were performed using appropriate biosafety measures. Blood samples were obtained by puncture, and they were collected without EDTA and heparin for the serum diagnostic with microscopic agglutination test (MAT) and for the detection of *Leptospira* DNA using real-time PCR; also kidney tissue samples were obtained aseptically for culture isolation of leptospires. A total of 34 wild animals (16 *R. norvegicus,* 11 *D. albiventris,* 6 *C. villosus* and 1 *L. griseus)* were chosen, analyzed and studied by the four mentioned techniques.

a. Isolation procedures of Leptospira by direct culture renal tissue: Kidney tissue from each animal was homogenized aseptically into transport medium (buffered solution at pH 7.2, containing 200 µg/ml of 5-fluorouracil as selective agent) for 2 hr. This suspension was diluted (1:10 and 1:100) with sterile phosphate-buffered saline, and 0.5 ml of each dilution was inoculated in EMJH liquid medium. Cultures were incubated at 28 C for 90 days and leptospiral growth was monitored weekly using dark field microscopy. The processing techniques and culture of the renal tissue, as the dilutions of the suspensions were made according to the Methods Manual for Laboratory Leptospirosis[15].
b. Microscopic agglutination test (MAT): Test was carried out with a battery of 10 *Leptospira* serovars (*L. interrogans* serovars Canicola, Hardjo, Hebdomadis, Icterohaemorrhagiae, Pomona, Pyrogenes, and Wolffi; *L. borgpetersenii* serovars Castellonis and Tarassovi; and *L. kirschneri* serovar Grippotyphosa) maintained in Ellinghausen-McCulough-Johnson-Harris (EMJH; Difco Laboratories, Detroit, Michigan USA) medium. The serum titer was the highest dilution which agglutinated 50% of the antigen and titers equal to or higher than 1:50 were considered as positive. Every serum was considered positive if it reacted with at least 50 % of clumped-together leptospires or non-existing to a final dilution of 1:50. In case of positive sample, the following dilutions were performed in geometric progression of 2 (1:50, 1:100, 1:200, etc.).
c. Real-time PCR technique to detect Leptospira DNA from serum samples: DNA extraction was performed by means of the commercial kit AxyPrep Multisource Genomic DNA Purification (Axygen, Tewksbury MA, USA). DNA present in each extraction was quantified by the Quantit PicoGreen dsDNA Assay (Invitrogen, Carlsbad, CA, USA) kit quantified with the aim of corroborating the correct DNA extraction. For the detection of *Leptospira* DNA, real-time PCR reactions were performed, using primers Lepto F y Lepto R, previously described by Smythe et al, (2002), without the use of a specific probe, which generate an amplification product of 87 bp from Leptospiral DNA (Table 1). Real-time PCR reactions

were performed in a Rotor Gene Q thermocycler (Qiagen Hilden, Germany), in a final volume of 20µl, using EvaGreen as fluorescent intercalating dye (KAPA HRM FAST, Biosystem Woburn, USA). The cycling program for the primers Lepto F and R that amplify a fragment of 87 bp from the 16S gene of *Leptospira*[16] consisted in an initial denaturalization of 3 minutes and 45 cycles of 15 seconds at 95°C, 20 seconds at 50°C and 20 seconds at 72°C. After the amplification, a melting curve was performed, being the specific melting temperature of 87°C. In both detecting reactions, were considered positive those samples with values of Ct (Cycle threshold) lesser than 40, in which the specific amplification of the PCR product by the analysis of melting was verified.

d. Sensibility and index of correlation among proofs: For the calculation of sensitivity, every positive individual for any of the used techniques was considered sick. The concordance index was used to determine the overall proportion of agreement between tests, and was obtained in the following way: CI Positive Agreements + Negative Agreements X 100/Total of Test.

Results

A total of 19 (56%) wild animals were positive in at least one of the techniques employed. By means of the test of microscopic agglutination, an 8.8% of positives samples was detected (3/34); being *R. norvegicus* (2/16) and *L. griseus* (1/1) the positive animals. The obtained titles were of 1:50 for *L. interrogans* serovar Canicola (3/3), *L. borgpetersenii* serovar Castellonis (2/3) and *L. kirschneri* serovar Grippotyphosa (1/1). Table 2 shows the distribution of wild animals according to capture area and sex, and comparison of results obtained with the techniques used.

The pathogenic leptospires were isolated in 2 of 34 samples of renal tissue (6.9%) from *R. norvegicus* trapped in peri-urban area of Tandil, Buenos Aires province*;* stocks were recovered right after 18 to 27 days of development in the cultivation environment.

In the case of serum samples studied by real-time PCR, it was detected 17/34 positive animals (50 %). It was positive in *R. norvegicus* (7/34), *D. albiventris* (6/34) and in *C. villosus* (4/34). The positives rodents were trapped in peri-urban and rural area of Azul and Lamadrid, Buenos Aires province; the *D. albiventris* and *C. villosus* were captures in rural area of Azul, Buenos Aires province.

Table 3, 4 shows that the index of concordance between proofs varied according to the technique and the sample used: MAT and real-time PCR in serum (CI: 47 %), MAT and Culture (CI: 91.2 %), and Culture with real-time PCR in serum (CI: 56 %).

Discussion

The positivity rate varied in particular according to the technique used, with the real-time PCR who detected the highest number of positive samples, and had a higher (85%) sensitivity to MAT (15%) and culture (10%). In this study the sensitivity of culture and MAT were low compared to PCR. Culture has low sensitivity because the isolation of this bacterium from clinical samples is technically demanding. Importantly, the stage of disease in which animals and the type of sample studied influences the sensitivity of the techniques found. According to what has been mentioned, the concordance rates varied between trials, obtaining a concordance of 91.2% for MAT and Culture (Table 4), 56% for culture and real-time PCR in the serum (Table 5) and 47% for MAT and real-time PCR (Table 3). The higher concordance observed between MAT and Culture is caused mainly because both techniques require a period of evolution of the disease in which circulating antibodies are observed, and there is invasion of leptospires in the renal tissue. By contrast, the MAT and real-time PCR assays (sera) and Culture with real-time PCR were the least coincident, with matches of 47% (Table 3) and 56% (Table 5) respectively. It is estimated that these results may be due to in the period of bacteremia (presence of *Leptospira* DNA) observed no circulating antibodies, or titles are not detectable by MAT. In previous work we have observed that in wild animals, especially *R. norvegicus*, with positive isolates from renal tissue samples was not possible

Table 1. Primers used in this study, sequence, amplicon size and reference

Primer	Sequence 5´- 3	Amplicon size (bp)	Reference
Lepto F	CCCGCGTCCGATTAG	87	Smythe et al, 2002
Lepto R	TCCATTGTGGCCGRACAC		

Table 2. Distribution of wild animals according to capture area and sex, and comparison of results obtained with the techniques employed

Area	Wild animals	Sex	MAT	Kidney culture	Serum RT- PCR
Peri-urban	*R. norvegicus*	M	Negative	Positive	Positive
Peri-urban	*R. norvegicus*	H	1:50 Grippotyphosa-Canicola-Castellonis	Positive	Positive
Peri-urban	*R. norvegicus*	H	1:50 Canicola-Castellonis	Negative	Negative
Peri-urban	*R. norvegicus*	H	Negative	Negative	Positive
Peri-urban	*R. norvegicus*	H	Negative	Negative	Negative
Peri-urban	*R. norvegicus*	H	Negative	Negative	Positive
Peri-urban	*R. norvegicus*	H	Negative	Negative	Negative
Peri-urban	*R. norvegicus*	M	Negative	Negative	Negative
Peri-urban	*R. norvegicus*	H	Negative	Negative	Negative
Rural	*D. albiventris*	H	Negative	Negative	Negative
Rural	*C. villosus*	M	Negative	Negative	Negative
Rural	*R. norvegicus*	H	Negative	Negative	Negative
Rural	*R. norvegicus*	H	Negative	Negative	Positive
Rural	*R. norvegicus*	H	Negative	Negative	Positive
Rural	*C. villosus*	M	Negative	Negative	Positive
Rural	*D. albiventris*	H	Negative	Negative	Negative
Rural	*D. albiventris*	M	Negative	Negative	Negative
Rural	*D. albiventris*	M	Negative	Negative	Positive
Rural	*D. albiventris*	M	Negative	Negative	Positive
Rural	*D. albiventris*	M	Negative	Negative	Positive
Rural	*L. griseus*	H	1:50 Canicola	Negative	Negative
Rural	*C. villosus*	H	Negative	Negative	Negative
Rural	*D. albiventris*	M	Negative	Negative	Negative
Peri-urban	*R. norvegicus*	H	Negative	Negative	Negative
Peri-urban	*R. norvegicus*	H	Negative	Negative	Negative
Peri-urban	*R. norvegicus*	H	Negative	Negative	Negative
Peri-urban	*R. norvegicus*	H	Negative	Negative	Positive
Rural	*C. villosus*	M	Negative	Negative	Positive
Rural	*C. villosus*	M	Negative	Negative	Positive
Rural	*D. albiventris*	H	Negative	Negative	Positive
Rural	*D. albiventris*	H	Negative	Negative	Positive
Rural	*D. albiventris*	H	Negative	Negative	Negative
Rural	*D. albiventris*	M	Negative	Negative	Positive
Rural	*C. villosus*	M	Negative	Negative	Positive

Table 3. Comparison of MAT and PCR results from serum samples

Test	MAT positive	MAT negative	Total
PCR positive	1	16	17
PCR negative	2	15	17
Total	3	31	34

Concordance Index: 47%.

Table 4. Comparison of MAT and Culture results from renal tissue samples

Test	MAT Positive	MAT Negative	Total
Culture positive	1	1	2
Culture negative	2	30	32
Total	3	28	34

Concordance Index: 91.2%

to detect the presence of antibodies by MAT (with titles cutting 1:20 and 1: 50). It is possible that the number of serovars used as antigen in MAT (10 serovars) was limited[2].

Using real-time PCR from serum sample, we obtained 50% of positive animals, associated with an early phase of the disease stage (acute leptospirosis). This would explain the one hand the presence of negative results with other techniques (MAT and Culture from renal samples), which try to detect animals in late-stage disease. All positive rodents (*R. norvegicus*) were captured from peri-urban as rural area, and these carriers' animals are considered major epidemiological source of transmission in this region. However, in this study other wildlife such as weasels and armadillos were positive by real-time PCR from serum samples, and even these species have proven to be carriers of *Leptospira* in Argentina. Real-time PCR in serum proved to have a high sensitivity being an important tool for the diagnosis of leptospirosis, especially in the acute phase of disease, in which the other techniques often provide negative results. Although the isolation of leptospires from clinical samples is strong evidence to confirm the diagnosis, identification of serotypes extending in a particular geographic region, meet the pathogenicity and virulence, and to use them as antigens in MAT for epidemiological studies.

Conflicto de intereses

No existe.

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