Original Article

Usefulness of real-time PCR assay targeting *lipL32* gene for diagnosis of human leptospirosis in Uruguay

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Abstract
Introduction: Assays based on DNA amplification can provide information that contributes to the initial management of patients with leptospirosis. However, these have not been adopted in Uruguay. Our aim was to evaluate the performance of the lipL32 real-time PCR (qPCR) for diagnosis of leptospirosis.
Methodology: We analyzed by microscopic agglutination test (MAT) and lipL32 qPCR serum samples from 183 patients with suspected leptospirosis. To establish the analytical sensitivity of the qPCR, experimentally spiked samples with known amounts of Leptospira interrogans were analyzed.
Results: The analytical sensitivity of the qPCR was 10^2 leptospires/mL. In 98 patients MAT results were negative meanwhile 85 showed positive reactions, revealing acute infections. Twenty six acute-phase sera of these 85 patients showed a positive signal by qPCR (diagnostic sensitivity 30%). In these patients the average time between onset of symptoms and collection of the first sample was 8 days. In patients with negative results for qPCR and positive MAT results (n=59) the average interval between onset of symptoms and collection of the first sample was 13 days. The qPCR did not yield false positive results.
Conclusions: The qPCR had a lower diagnostic sensitivity than MAT and a higher cost. However, it allowed to make an early diagnosis in 26 patients. In patients with confirmed acute infections and negative results by qPCR, more than 8 days had elapsed between the onset of the illness and extraction of the first serum sample. Our data support that the qPCR from sera have clinical utility within the first week of illness.

Introduction

Leptospirosis is a worldwide zoonotic disease caused by pathogenic spirochetes belonging to the genus *Leptospira*. Currently, nine pathogenic species subdivided into more than 200 serovars are recognized within the genus [1,2,3].

Leptospira interrogans is the main species associated with human leptospirosis. Transmission occurs through direct or indirect contact with urine of infected domestic or wild animals (especially cattle and rodents) or by exposure to contaminated water or moist soil [4].

Leptospirosis is an acute febrile disease with a broad spectrum of nonspecific symptoms and signs which makes diagnosis difficult when based only on clinical data. The disease can be particularly confused with dengue fever and dengue hemorrhagic fever [5,6] and it is thus important that there are suitable laboratory diagnostic tests available to enable appropriate treatment of patients.

Definitive diagnosis can be made by isolation and identification of the bacteria from tissue or body fluids (e.g. blood, cerebrospinal fluid) but this requires prolonged periods of incubation and a set of absorbed antisera to establish the serovar of the recovered strains [7].

The "gold standard" for serological diagnosis of leptospirosis is the microagglutination test (MAT): a time consuming assay requiring viable bacteria and experienced personnel [4,8]. The MAT can also show false negative results in the early stages of infection [9].

A variety of rapid serological tests other than MAT have been developed for laboratory diagnosis including several enzyme-linked immunosorbent

assays, a macroscopic slide agglutination test and indirect immunofluorescence (IF) procedures [10-14].

Real-time PCR (qPCR) is a powerful technique routinely applied as a diagnostic tool in different fields, including clinical microbiology [15]. In Uruguay, this platform is currently used by several laboratories to study viruses and bacteria responsible for diseases in people and animals.

There are several qPCR assays described for detecting pathogenic and non-pathogenic *Leptospira*. Some amplify particular sequences of genes that are universally present in bacteria like the 16S rRNA, while others target genes such as *lipL32* which is considered to be restricted to pathogenic species [16,17]. Although expensive, these assays provide a rapid diagnosis and can be positive before the appearance of antibodies detected by MAT, IF or ELISA tests.

The aim of this study was to evaluate the performance of the *lipL32*qPCR with SYBR green for early diagnosis of human leptospirosis in Uruguay.

Methodology

Bacterial strain and culture conditions

Leptospira interrogans serovar Pomona strain Pomona (confirmed by multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) [18] from the collection of the Hygiene Institute (HI) was used as a positive control in all DNA amplification reactions and also to create positive samples by spiking. *L. interrogans* serovar Pomona was maintained in Fletcher semisolid medium (Becton Dickinson, Le Pont de Claix, France) and grown aerobically at 30°C in Ellinghausen–McCullough–Johnson–Harris (EMJH) liquid medium (Becton Dickinson, Le Pont de Claix, France) [19].

Patient sera, collection and storage

Between January 2012 and April 2013 we used MAT and *lipL32* qPCR to test 235 serum samples from patients with clinically suspected leptospirosis that were sent to our laboratory for routine diagnosis.

Blood was obtained by venipuncture; serum was separated and stored at -20 ° C until tested.

We also used both procedures to analyze sera from 20 patients with confirmed syphilis and 15 with acute hepatitis B virus infection.

Clinical and epidemiological information of each patient with suspected leptospirosis was obtained from the medical records.

Micro Agglutination Technique (MAT)

MAT was performed on all sera (acute and convalescent-phase samples) with a two-step procedure [4,8]. Each serum was initially diluted 1:25 in saline and 100 µL was mixed in plastic plate wells with 100 µL of each of 20 live cultures of *Leptospira* serovars frequently detected or cross-reacting with serovars common to our region. The live liquid cultures were 7-15 days old and adjusted to a 0.5 McFarland standard scale. After one minute of rotary mixing, the plastic plates were incubated under standard conditions. A positive reaction was observed when microclumps could be detected and less than 50% of the live *Leptospira* were not agglutinated. Positive and negative control sera were included in each plate. In a second step, serovars that produced a positive result and did not show auto-agglutination or false positive results were tested against serial dilutions of the patient´s serum to determine titers.

Sample contamination

Serum, plasma and whole blood samples obtained from a patient with two consecutive negative MATs were spiked with known amounts of *L. interrogans* serovar Pomona. Organism counts were carried out in duplicate by two investigators (SG, JPG) using a 10-15 day old culture in EMJH and a Petroff–Hausser chamber (Fisher Scientific International, Hampton, New Hampshire, USA) and the culture concentration adjusted to 10^8 bacteria/mL. Bacterial suspensions were spiked into whole blood, plasma and serum so that the final concentrations were 10^5, 10^4, 10^3, 10^2 and 10^1 leptospires/mL.

DNA extraction

A commercial kit (GE, illustra™, Healthcare Life Sciences, Sweden) was used to extract the DNA from 300 µL of the patients' sera and from the spiked samples following the manufacturer's instructions. A known negative serum sample was used as a negative control in each DNA extraction procedure.

Real Time PCR (qPCR)

We used the procedure previously described by Stoddard *et al.* [20] and later modified by Bourhy *et al.* [21], to study acute-phase serum from patients and experimentally spiked samples.

Briefly, 4 µL of DNA from each sample were amplified in a 20-µL reaction mixture containing 1X Rotor-Gene FAST SYBR Green PCR Master Mix (QIAGEN, Maryland, USA) 4 mM Cl_2Mg and 0.2 µM each primer lipL32-45F

Figure 1: Limits of detection of a *lipL32* q-PCR assay using DNA extracted from serum, plasma and whole blood samples spiked with known amounts of *Leptospira interrogans* serovar Pomona strain Pomona. Each point represents the mean of 3 replicates assays. The equation of the line and R^2 are displayed
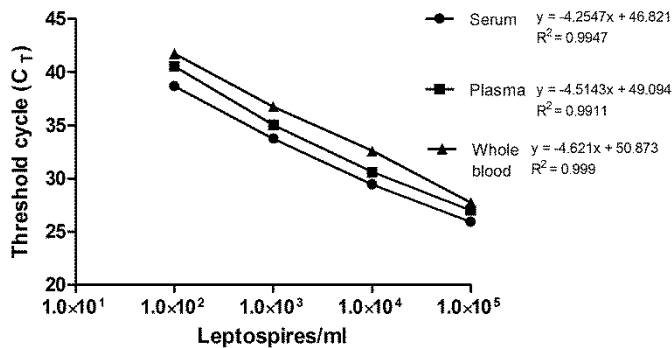
(5´AAGCATTACCGCTTGTGGTG 3´) and lipL32-Rb (5´ GAACTCCCATTTCAGCGAT 3´) (SBS Genetech Co., Ltd, Mainland, China). To detect the presence of amplification inhibitors, we also included RNAseP3F (5´ CCAAGTGTGAGGGCTGAAAAG 3´) and RNAseP3R (5´ TGTTGTGGCTGAACTATAAAGG 3´) primers at final concentrations of 0.2 µM. This primer pair targets a 51 bp fragment corresponding to the *rnaseP* eukaryotic gene.

Amplifications were performed using a Rotor-Gene 6000 system (Corbett Life Science, Sydney, Australia) and the protocol was of 15 minutes at 95º C, followed by 45 cycles of amplification (95º C for 10 seconds , 55º C for 15 seconds and 72º C 20 seconds).

A melting curve with a ramp speed of 1 ºC/s between 65° C and 95° C was determined with a reading every 0.2° C.

In each run, we included a tube with DNA obtained from serum spiked with 10^5 leptospires/mL as a positive control and also another one with a heat-extract from *L. interrogans* serovar Pomona. As a negative control we used DNA extracted from serum from an uninfected patient.

To establish the analytical sensitivity of the qPCR, each experimentally spiked sample (whole blood, plasma and serum) was run in triplicate.

Results

Only 183 of the 235 patients had acute and convalescent (obtained 10-15 days later) serum samples we could test by MAT .

In 98 of 183 patients the MAT results were negative with 85 being positive and supporting a diagnosis of acute infection (MAT-/+).

The qPCR could detect down to 10^2 leptospires/mL in spiked serum, plasma and whole blood samples with detection occurring at lower cycle numbers in the case of spiked serum samples (Figure 1).

Sera from 20 patients with syphilis, 15 with acute hepatitis B infections, and 98 negative on consecutive MATs were negative by q-PCR assay (specificity 100%). All samples were positive for the *rnaseP* gene (Tm value between 77 and 78 ° C).

Only 26 acute-phase sera from the 85 (acute negative / convalescent positive) sera that were positive by MAT were positive by qPCR (sensitivity 30%). All had a Tm between 84 and 85 ° C, corresponding to the expected Tm for the amplification fragment of the *lipL3* gene.

In the positive patients the mean delay between onset of symptoms and collection of the first sample was 8 days. One of these patients died and another one showed severe illness with impairment of liver and kidney function.

In patients with negative results by qPCR but positive by MAT (n = 59), the mean delay between onset of symptoms and collection of the first sample was 13 days. All these samples showed positive results to the *rnaseP* gene. None of these patients died and none had severe disease.

Male patients accounted for more than 95% of the 85 MAT confirmed cases of leptospirosis and they were mainly rural workers aged 20 to 40 years. Ninety percent of patients presented with fever, fatigue, myalgia and headaches while conjunctival hyperemia was observed in only 30%.

Discussion

In Uruguay, leptospirosis is a reemerging neglected disease and represents a major concern to public health. We estimate that at least 500 new cases occur per year, with an approximate incidence of 15 cases per 100,000 inhabitants [22]. Since 2000, MAT has been routinely used in our laboratory for the diagnosis of leptospirosis. However, MAT enables only retrospective diagnoses which do not help with the immediate management of patients [23]. Also, in many patients a convalescent-phase serum sample is not collected and the diagnosis is uncertain. In our study, for example, we had a second serum sample from only 183 of the 235 patients, complicating laboratory confirmation of infections [22]. This

situation led us to consider the diagnostic performance of qPCR and other tests such as IgM-IF that enable the rapid laboratory diagnosis of leptospirosis by analyzing a single serum sample obtained during the acute-phase of the illness.

In our hands, the qPCR assay showed high sensitivity, detecting 10^2 bacteria per mL in DNA extracted from spiked serum, plasma or whole blood samples. This value is higher than that reported by Stoddard *et al.* for serum samples [20] but these workers spiked whole blood before it clotted and the serum was separated for DNA extraction and qPCR. As leptospires can be retained in the clot this was probably the reason they found lower numbers of bacteria in the serum samples they tested.

The sensitivity of the qPCR we used in identifying infected individuals was only 30%, lower than that of the MAT. Although this finding is consistent with previous reports, our figure is lower than the values close to 50% reported by other authors [24-26].

This low diagnostic sensitivity of our qPCR may be due to several factors: most leptospirosis cases are mild and develop only a low bacteriema of $<10^2$ leptospires/mL; the samples are often obtained when antibodies are present, several days after the onset of the illness or after starting treatment with antibiotics. These factors are known to decrease the chances for recovering leptospires from the blood and also for obtaining positive results by *in vitro* DNA amplification methods [4,20,25,26]. In most patients with MAT -/+ reactions and negative results by qPCR (n=59) more than 13 days had elapsed between the onset of the disease and the collection of a first serum sample. Further, in many of these patients detection of IgM antibodies by immunofluorescence assay was positive (data not shown). Finally, the samples we used had been frozen and stored at -20ºC which could decrease the number of leptospires detectable in the samples.

Overall, however, the q-PCR enabled us to make an early and rapid diagnosis in 26 of 85 patients (2 with severe disease) before a positive MAT could be obtained. Further, the assay did not give any false positive results.

Our study suggests that the qPCR is useful within the first week of clinical disease and that sera may be better than whole blood samples for the diagnosis of acute human leptospirosis, as reported previously [27-29]. As the reliability of the test decreases with time after onset of signs, we believe that efforts should be made to raise awareness amongst health workers that as soon as there is a clinical suspicion of leptospirosis

blood/serum samples should be submitted to increase the possibility of accurate diagnoses. As suggested by Agampodi *et al.* [28], our qPCR using SYBR green appears to be a rapid, specific and less expensive method than the TaqMan assay for use in laboratories that process a relatively large number of samples per month and have funding constraints. In such laboratories the suggested diagnostic algorithm would be to obtain samples (especially from sick men, between 20 and 40 years old and rural workers) within the first seven-eight days of the onset of symptoms. The samples should comprise blood for culture and serum for performing qPCR and IgM detection. If the results are negative by qPCR and IgM investigation, a second serum sample should be obtained in 10-15 days for MAT. Even if the initial results are positive through qPCR or IgM detection, a second sample should be requested after 15 to 20 days to confirm diagnosis by MAT.

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