Diagnostic Microbiology and Infectious Disease 78 (2014) 343–346

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Evaluation of nested polymerase chain reaction for the early detection of *Leptospira* spp. DNA in serum samples from patients with leptospirosis

Roberta Morozetti Blanco, Eliete Caló Romero *

Center of Bacteriology, Instituto Adolfo Lutz, Av. Dr. Arnaldo, 355, CEP 01246–902, São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 22 August 2013
Received in revised form 13 December 2013
Accepted 13 December 2013
Available online 21 December 2013

Keywords:
Leptospirosis
Leptospira spp
Molecular diagnosis
Serum
Nested PCR

ABSTRACT

The aim of this study was to analyse the nested polymerase chain reaction (nested PCR) in human serum samples of patients with clinical manifestations of leptospirosis. The cases of leptospirosis were defined by the microagglutination test (MAT). The samples were collected in 2010. Of 1042 serum samples collected from 521 patients, 28 (5.4%) were considered positive cases of leptospirosis, and 493 (94.6%) were negative. Twenty-three confirmed cases had no MAT-detectable antibodies in the acute sample (mean of 5.6 days after onset). Nested PCR was positive in 22/23 (95.7%) patients during the acute phase of the disease, with negative results by MAT. Nested PCR was negative in all convalescent serum samples with positive results by MAT. All negative cases of leptospirosis were negative by nested PCR. The nested PCR is an alternative diagnostic tool for early detection of leptospires in sera during the first 7 days of the disease.

1. Introduction

Leptospirosis is a global zoonotic disease caused by pathogenic members of the genus *Leptospira*. In Brazil, the disease is endemic and is often related to heavy rainfall and flooding, with case fatality rates as high as 15% (Ko et al., 1999). The severity of this acute febrile illness varies considerably from mild to rapidly fatal, and the wide spectrum of symptoms makes clinical diagnosis very unreliable. Appropriate treatment is most effective if started early and decreases the risk of severe complications such as pancreatitis, vasculitis, and pulmonary hemorrhages. Culture methods are well established for leptospires but are technically demanding and time consuming, often taking several weeks to achieve observable growth (Faine et al., 1999). Serology remains the main diagnostic tool, and the microscopic agglutination test (MAT) is the standard method for the diagnosis of leptospirosis. However, this method has the disadvantages of requiring acute and convalescent sera and is tedious and time consuming to perform. Also, the interpretation of the results is subjective (Romero et al., 1998b), and the sensitivity is low (Bajani et al., 2003; Levett and Branch, 2002). Several approaches, such as real-time polymerase chain reaction (PCR), have been developed for early detection of leptospires (Ahmed et al., 2009; Bedir et al., 2010; Bourhy et al, 2011; Fearnley et al., 2008); however, serum samples are not the ideal material for detection of leptospires by this method (Stoddard et al., 2009). Since serum is commonly collected for serologic testing and is readily available in diagnostic laboratories, the aim of this study was to analyse whether nested PCR specific for *Leptospira* spp. could be used for early detection of leptospires in sera as an alternative to conventional diagnostic methods.

2. Materials and methods

2.1. Leptospira and growth conditions

Leptospira interrogans serovar Copenhageni strain M20 was maintained in liquid Ellinghausen-McCullough-Johnson-Harris medium supplemented with 10% enrichment medium (Difco, Detroit, MI, USA) and was incubated at 28 °C for 7 days. This strain was used to spike the serum samples.

2.2. Patient serum samples

A total of 1042 serum samples obtained from 521 patients with suspected leptospirosis (an acute and convalescent phase sera for each patient) were analysed prospectively in a blinded manner. The samples were collected in 2010. Samples were sent to the Adolfo Lutz Institute, São Paulo, SP, Brazil, for MAT, PCR, and nested PCR testing. According to the MAT results, the samples were classified into 2 categories as follows:

- Group 1 (n = 493 patients): negative cases of leptospirosis
- Group 2 (n = 28 patients): confirmed cases of leptospirosis

This study was approved by the Ethical Committee for Research in Adolfo Lutz Institute of São Paulo.

* Corresponding author. Tel.: +55-11-30682897.
E-mail addresses: eliete_romero@yahoo.com.br, ecromero@ial.sp.gov.br (E.C. Romero).

0732-8893/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.diagmicrobio.2013.12.009

2.3. MAT

The microscopic agglutination test was performed according to the standard method (Faine et al., 1999) with the following reference serovars representing the serogroups of *Leptospira* spp. most frequently found in São Paulo, Brazil (Romero and Yasuda 2006; Romero et al., 2003; Romero et al., 2011): Australis, Autumnalis, Bataviae, Butembo, Canicola, Castellonis, Celledoni, Copenhageni, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc, Pomona, Pyrogenes, Shermani, Tarassovi, and Wolfii. A sample was considered reactive if it agglutinated more than 50% of the leptospires. A case of leptospirosis was confirmed when 2 samples had a titer rise of >2 dilutions between acute and convalescent samples or when had a minimum titer of 1:800. A negative case of leptospirosis was defined as having negative MAT results in serum samples. The highest sample dilution showing 50% agglutination was identified as the predominant serogroup.

2.4. Storage of serum samples

To assess whether the storage of samples increased the inhibitory nature of the sera, we froze serum samples spiked with *Leptospira* DNA in triplicate at 1, 7, and 30 days. All aliquots were thawed on ice to avoid nuclease activity and degradation of possible *Leptospira* DNA.

2.5. DNA extraction

DNA was extracted from 100 μL of each serum sample. Different DNA extraction methods were evaluated to determine which was optimal for use with the PCR and nested PCR assays. DNA was extracted from the reference strain *L. interrogans* serovar Copenhageni by the boiling method (Romero et al., 1998a), by phenol-chloroform (Sambrook and Russell, 2001), and according to the manufacturer's instructions using QIAamp DNA mini Kit (Qiagen, Hilden, Germany). One sample of each extraction was used immediately after the extraction. One sample was stored at −20 °C, and another was stored at 4 °C until use in PCR and nested PCR (1–30 days after extraction).

2.6. PCR and nested PCR

All the PCR assays were performed in 3 separate rooms (clean room, sample addition room, and dirty room), and adequate care was taken to prevent contamination by carry-over amplicons between the first (PCR) and the second amplification (nested PCR). Primers corresponding to nucleotides 38–57 (primers A and B) and 348–368 (primers C and D) in the primary structure of the *L. interrogans rrs* (16S) (Mérien et al., 1992) gene were used for PCR and nested PCR, respectively. The specificity of the primers was previously analysed (Mérien et al., 1992; Romero et al., 1998a). Briefly, a 25-μL reaction mixture contained 10× assay buffer, 200 μmol/L of each deoxyribonucleotide triphosphates (dATP, dGTP, dCTP, and dTTP), 1 μmol/L of each primer, 1 unit of Taq DNA Polymerase (Promega, Madison, WI, USA), 2 mmol/L of $MgCl_2$, and 10 μL of DNA extracted from the serum samples. The amplification reaction was performed with a programmable thermal cycler (Perkin–Elmer/Cetus, Norwalk, CT, USA). PCR conditions were as follows: an initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 1 min followed by annealing at 63 °C for 1.5 min, extension at 72 °C for 2 min, and a final elongation step at 72 °C for 10 min. All samples were further tested with primers C and D by nested PCR. The reaction was performed under the same conditions as PCR, using 1 μL of the first PCR reaction as the template with primers C and D. The amplification was done for 35 cycles using the same cycling parameters as the PCR. Serum samples from healthy donors with negative results by MAT as well as water (Milli-Q) were used as negative controls. Serum samples spiked with DNA from *L. interrogans* serovar Copenhageni strain M20 were used as the positive control. Each run of the assay included 2 negative controls and a positive control. Each specimen was tested in duplicate.

2.7. Analysis of amplified products

The amplified products were subjected to electrophoresis on a 1.5% agarose gel using 10 μL of the product. After electrophoresis, the gel was stained with ethidium bromide, visualized, and photographed under UV light.

2.8. Detection limit determination

The limit of detection of the assay was defined as the lowest number of leptospires that could be visually detected on a gel using 100 μL of healthy donor serum spiked with 10^0–10^6 cells of leptospires. Serial 10-fold dilutions were prepared from DNA extracted by QIAamp DNA Mini Kit (Qiagen) from leptospires in culture medium and spiked serum samples. In addition, the limit of detection of *Leptospira* spp. by PCR and nested PCR was determined by testing different amounts of DNA template from 100 ng to 10 fg.

2.9. Performance of the PCR and nested PCR assay

The sensitivity of the PCR and nested PCR assay was calculated based on case confirmation of leptospirosis by MAT. The specificity was calculated based on group 1.

3. Results

Since all methods of extraction had similar results, the boiling method was chosen for DNA extraction from the clinical samples. The detection limit of the assay was 1 pg of DNA by PCR and 10 fg by nested PCR. The lowest number of leptospires that was amplified and visually detected was 10^1 by PCR and 10^0 by nested PCR. No specific amplification was obtained with clinical samples from healthy donors. From 521 patients, 493 (94.6%) were negative to leptospirosis (group 1), and 28 (5.4%) patients (group 2) were confirmed cases of leptospirosis. All negative cases of leptospirosis were negative by both PCR and nested PCR. The specificity was 100%. Twenty-three confirmed cases had no MAT-detectable antibodies in the acute sample (mean of 5.6 days after onset). From these, 1 serum sample was negative by both tests. Three serum samples were negative both by PCR and nested PCR with MAT-detectable antibodies in the acute sample (mean of 6 days after onset). Table 1 shows the results of PCR, nested PCR, and MAT with regard to days post onset. Single PCR was less sensitive than nested PCR. Positive results by PCR with primers A and B were found in 4/28 (14.3%) patients during the acute phase of the disease, whereas nested PCR assay was positive in 24/28 (85.7%) cases of leptospirosis. Positive results by nested PCR were found in 22/23 (95.7%) patients in the acute phase samples, which were negative by MAT. The calculated sensitivities of the PCR and nested PCR assays were 14.3% (95% confidence interval [CI]: 4.7–33.6%) and 85.7% (95% confidence interval [CI]: 66.4–95.3%), respectively. For convalescent sera (mean of 13.6 days after onset), neither PCR nor nested PCR was positive. Serum samples stored at 1 and 7 days showed the same results by both PCR and nested PCR, whereas serum samples stored at 30 days were negative by both PCR and nested PCR. On the other hand, all extractions stored at −20 °C and 4 °C showed the same results by PCR and nested PCR until 30 days after extraction. The samples that were used immediately after extraction and stored at −20 °C and 4 °C also showed similar results.

4. Discussion

The present study reports the utility of nested PCR in detecting leptospiral DNA in serum samples. The sensitivity of the nested PCR