Following the course of human leptospirosis: evidence of a critical threshold for the vital prognosis using a quantitative PCR assay

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Abstract

In order to follow the course of acute human leptospirosis, an ELISA microtiter plate hybridization method was developed for the quantitative determination of Leptospira spp. in biological samples after PCR. The biotin-labelled amplified product (331 bp from the rrs gene) was hybridized with a complementary capture probe covalently linked onto aminated polystyrene wells, and detected using a colorimetric reaction. The mean detection limit was 50 copies per 10 μl. In a prospective study of human leptospirosis cases, we obtained evidence that a density of 10^4 leptospires per ml of blood is a critical threshold for the vital prognosis of the patients. The practicability of the method makes it suitable for use in tropical areas for multicentric studies. Such studies could lead to a better knowledge of the natural history of the human disease. The method is also suitable for experimental evaluation of improved antibiotic treatments for leptospirosis. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Leptospirosis; Leptospira interrogans; Quantitative polymerase chain reaction; Pathogenesis

1. Introduction

Leptospirosis, which is caused by pathogenic species of the genus Leptospira, is a worldwide zoonanthroponosis affecting wild and domestic animals and humans. Because of the broad range of clinical symptoms, the diagnosis of human leptospirosis remains difficult [1]. Laboratory confirmation is, therefore, essential for effective therapy and for epidemiological surveys. However, standard methods (i.e., microscopic agglutination test and culture in EMJH medium) are time-consuming and lack sensitivity [1]. A combined polymerase chain reaction (PCR)-hybridization test has previously been proposed for qualitative detection of a specific target DNA sequence (16S RNA gene) of Leptospira [2]. This did not, however, allow the evaluation of bacterial density [3].

Quantification of a pathogen contributes to the knowledge of the natural history of the disease and may provide useful information for the monitoring of patients. Various colorimetric quantitative PCR techniques in microtiter plates have been described for many micro-organisms [4–6]. In leptospirosis, the kinetics of infection remain poorly investigated, and little is known about the pathogenesis of the human disease. As the majority of the human cases occur in tropical areas, a thorough study of the natural history of the disease requires tools to be available that are cost-effective as well as reliable.

In the present work, a rapid and simple microtiter plate hybridization assay was developed, for the quantification of pathogenic leptospires after PCR amplification with biotinylated primers. The capture probe was covalently linked onto aminated wells of microtiter plates, using carbodiimide as a coupling agent. This method was validated in a prospective study of blood and urine samples from confirmed cases of human leptospirosis.

2. Materials and methods

2.1. Preparation of DNA for standard curve

For the standard curve, various numbers of leptospires (serovar Icterohaemorrhagiae strain Verdun), ranging in
an geometric ratio of 10 from 20 to $2 \times 10^7$, were heated (in 100 µl sterile distilled water) for 10 min at 96°C, and a 10-µl aliquot (corresponding to 2 to $2 \times 10^6$ leptospires) was used for PCR.

2.2. Treatment of clinical specimens and DNA amplification

A total of 27 biological samples (blood, urine) submitted to our laboratory were obtained from 12 patients with biologically confirmed leptospirosis. The microscopic agglutination test was performed according to standard procedures [1]. Serologically confirmed cases were those showing seroconversion (negative first serum sample and a titer $\geq 1:100$ in the second sample) or a rise in titer of at least two dilutions between the acute and the convalescent phases. When available, clinical specimens were cultured in EMJH medium for primary isolation [1]. For the quantification of DNA in biological samples, the DNA from *Leptospira* spp. was purified according to Boom et al. [7]. 10 µl of eluted DNA in TE buffer were used for subsequent amplifications. A 331-bp fragment and its complementary probe (CD probe; 289 bp) of the 16S rRNA gene was amplified with 30 cycles and quantification was performed according to the standard curve. The sensitivity of the method was determined in the exponential part of the curve obtained with the calibrating DNA. The detection level was defined as the lowest concentration exceeding the zero dose precision and was estimated using the equation: cut-off = mean absorbance of negative sample + 3 × S.D.

3. Results

3.1. Standard curve

Serial dilutions corresponding to 2 to $2 \times 10^6$ leptospires were amplified with 30 cycles and quantification was performed. Absorbance values were plotted against the number of *Leptospira* cells, resulting in a typical sigmoid dose-response curve (Fig. 1). A linear exponential relationship was demonstrated within the range of $10^2$–$10^5$. The negative absorbance cut-off, defined as the mean absorbance obtained from three negative samples (TE buffer) plus 3 S.D., was used to determine the detection limit of the assay. In the following experiments, an absorbance of 0.06 was calculated as the cut-off value, corresponding to an initial number of 50 bacteria. The detection limit was 20-fold greater than that of gel electrophoresis, with which $10^3$ *Leptospira* cells were visually detected after amplification for 30 cycles [2]. The reproducibility of the method was determined in the exponential part of the standard curve by testing independently amplified samples.
highest PCR-estimated bacteremia with at least 10^4 leptospires ml^-1 of blood. Interestingly, these three patients showed the positive culture in EMJH medium (serogroup Icterohaemorrhagiae). Among the other six patients, two showed seroconversion (patients C and L), and one (patient G) had a significant rise in titer. In the three remaining cases (patients B, D and K), a significant follow-up was not possible, owing to the availability of a single serum (case B; high titer of 400) or to a too short period between the two serum samples (cases D and K).

3.2. Detection of Leptospira spp. in human samples

The results of our prospective study are summarized in Table 1. Ages of the 12 patients ranged from 17 to 73 years (mean 36); nine were male and three were female. Twenty-seven samples, including 23 blood specimens and four urines, were collected for testing. Leptospirosis was confirmed both serologically (rise in titer) and by positive culture in six cases (patients A, E, F, H, I and J). The six isolated strains were identified as belonging to serogroup Icterohaemorrhagiae. Among the other six patients, two showed seroconversion (patients C and L), and one (patient G) had a significant rise in titer. In the three remaining cases (patients B, D and K), a significant follow-up was not possible, owing to the availability of a single serum (case B; high titer of 400) or to a too short period between the two serum samples (cases D and K).

Three patients (D, F and H) were hospitalized in an intensive care unit and died of acute leptospirosis 2-4 days after admission. Two of these (F and H) gave a positive culture in EMJH medium (serogroup Icterohaemorrhagiae). Interestingly, these three patients showed the highest PCR-estimated bacteremia with at least 10^4 leptospires ml^-1 of blood (maximum 1.5×10^9 ml^-1 of blood, patient H). Antibiotic treatment was started on admission to hospital. For patient D, this antibiotic therapy had no effect on the level of PCR-estimated bacteremia, as an increase of two log occurred. Estimated bacteremia was significantly reduced in patients F and H, but the patients nevertheless developed hemorrhagic complications and died.

A weak PCR-estimated bacteremia (less than 10^4 leptospires ml^-1 of blood) was observed in the other nine patients (A, B, C, E, G, I, J, K and L) (maximum 2.7×10^3 ml^-1 of blood, patient K), all of whom recovered. When available, urines were always weakly positive (average of 2.5×10^3 leptospires ml^-1; patients A, B, C and G). In three of these patients (A, B, C), the level of leptospiral DNA in urine was higher than that in the blood. This supports the potential value of urine for early PCR detection of leptospires in humans [9].

4. Discussion

Quantification of viral and bacterial agents in biological samples using PCR amplification has value for clinical and research applications. These applications include the monitoring of treatment, pathogenesis studies, and standardization of assays between laboratories. Microtiter plate-based hybridization offers the most convenient method for detecting and quantifying PCR-amplified fragments. However, variations in the passive binding capacity of polystyrene surfaces limit the use of these commercially available supports in hybridization assays. The recently developed real-time PCR system has great potential. However, the spread of this technique is restricted by its very high cost. This is especially the case in tropical areas where human leptospirosis is endemic.

In the present study, we used direct immobilization onto CovaLink NH plates with carbodiimide condensation to covalently bind a capture probe, complementary to the diagnostic amplicon, onto polystyrene (secondary amino group). This step offers two main advantages compared with passive adsorption: the attachment is quite stable, even after 3 weeks of storage at 4°C, and the denaturation step can be performed directly in the microwells. Satisfactory reproducibility, sensitivity and specificity are obtained, and this colorimetric microtiter plate hybridization assay can be used for quantifying leptospiral DNA using standard laboratory ELISA equipment.

Following patients in this study allowed us to estimate the bacterial density in the blood during the course of the disease. In most cases the estimated density was around 10^2–10^4 leptospires ml^-1 (average on the first sample in the period D1–D4: 1.0×10^4 leptospires ml^-1) (Table 1). Septicemia in leptospirosis can be considered as moderate [1]. The clinical evolution of our cases indicates that a density of 10^4 leptospires ml^-1 of blood can be considered as a critical threshold for the vital prognosis of the patients. Indeed, the three patients with estimated bacteremia to this level all died. Evidence for the concept of a critical threshold relating to lethality was previously obtained using a mouse model of leptospiral infection [10]. In one case (patient F), a decrease in the estimated number of circulating leptospires under the threshold of 10^4 leptospires ml^-1 was observed, but this patient did not recover. This was consistent with the course of experimental disease in laboratory rodents. They may die even though leptospires have been cleared from the bloodstream, if lesions in target organs (kidneys, liver) can be demonstrated [1,11].

![Fig. 1. Amplification of Leptospira spp. DNA by PCR (30 cycles) and detection of amplified DNA by colorimetric microtiter plate hybridization. Data are presented with standard deviations. The dotted line represents the cut-off value.](Image)
In conclusion, the method described here can be applied to the rapid detection and quantification of leptospires in biological samples. The instrumentation developed for ELISA techniques can be used, allowing a large number of samples to be analyzed safely and conveniently. This quantitative PCR assay has two main potential applications. The first application is in studies of pathogenesis. Most current knowledge about the molecular determinants of pathogens comes from in vitro studies. However, bacterial growth in vivo changes during the course of infection, according to the target tissues and the host defences. In leptospirosis, little is known about the growth rates during infection in target organs in animal models [11]. Our data demonstrating the possibility of a critical threshold of PCR-estimated bacteremia in human disease are of significant interest, and would justify a multicentric prospective study. The other potential application is for the improvement of drug therapy. Leptospires are sensitive to a wide range of antibiotics, and the acquisition of new resistances has not been reported. However, standard antibiotic regimens using β-lactamins are not always effective in treating leptospirosis, as a persistent presence of leptospires has been observed in human patients [3,9]. Usual treatments may be unable to remove leptospires from the sites where they are protected from the immune response (meninges, eyes). Evaluation of various antibiotic regimens using animal models would help to define more efficient therapeutic strategies.

Table 1
Quantitative PCR, serologic and culture results for 12 leptospirosis patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)/sex</th>
<th>Day of sampling</th>
<th>Biological samples</th>
<th>Culture in EMJH medium</th>
<th>Serology</th>
<th>PCR estimation of number of leptospires ml⁻¹³</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>38/F</td>
<td>D7</td>
<td>blood + (sg Ictero)</td>
<td>100 2 x 10⁴</td>
<td>3.6 x 10³</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D10</td>
<td>blood nsc</td>
<td>3200</td>
<td>3.9 x 10³</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>57/M</td>
<td>D9</td>
<td>blood nsc</td>
<td>400</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D1</td>
<td>blood nsc</td>
<td>50</td>
<td>2.3 x 10³</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>73/M</td>
<td>D1</td>
<td>blood nsc</td>
<td>200</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D³</td>
<td>45/M</td>
<td>D3</td>
<td>blood nsc</td>
<td>100 6.1 x 10³</td>
<td>5.7 x 10⁵</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>24/M</td>
<td>D4</td>
<td>blood nsc</td>
<td>200 1.5 x 10⁵</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>F³</td>
<td>29/M</td>
<td>D3</td>
<td>blood + (sg Ictero)</td>
<td>100 5.2 x 10⁴</td>
<td>1.0 x 10²</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D7</td>
<td>blood nsc</td>
<td>12800</td>
<td>2.4 x 10²</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>35/M</td>
<td>D5</td>
<td>blood nsc</td>
<td>400 9.6 x 10¹</td>
<td>8.0 x 10¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>D9</td>
<td>blood nsc</td>
<td>3200</td>
<td>1.1 x 10²</td>
<td></td>
</tr>
<tr>
<td>H³</td>
<td>39/M</td>
<td>D17</td>
<td>blood + (sg Ictero)</td>
<td>100 1.5 x 10⁶</td>
<td>7.1 x 10⁴</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>18/M</td>
<td>D7</td>
<td>blood + (sg Ictero)</td>
<td>100 2.0 x 10²</td>
<td>7.9 x 10¹</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>30/F</td>
<td>D14</td>
<td>blood + (sg Ictero)</td>
<td>400 1.1 x 10²</td>
<td>8.6 x 10¹</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>29/F</td>
<td>D2</td>
<td>blood nsc</td>
<td>400 2.7 x 10³</td>
<td>5.4 x 10²</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>17/M</td>
<td>D2</td>
<td>blood nsc</td>
<td>800 3.0 x 10²</td>
<td>5.6 x 10¹</td>
<td></td>
</tr>
</tbody>
</table>

nsc: not subjected to culture. sg Ictero: isolated strain typed as from serogroup Icterohaemorrhagiae.

³D0: estimated day of onset of illness.
³Dead patient.
Acknowledgements

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References