Leptospirosis diagnosis by immunocapture polymerase chain reaction: a new tool for early diagnosis and epidemiologic surveillance

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A B S T R A C T

The aim of this study was to develop an immunocapture polymerase chain reaction (IC-PCR) protocol for leptospirosis. For the standardization of IC-PCR, polyclonal (AS) and monoclonal (MAb) antibodies against different serogroups and serovars of Leptospira were coupled to polystyrene plates. Human sera were artificially contaminated with leptospires and incubated on plates. The bacterial DNA was obtained and used in a multiplex PCR. Sensitivity was tested using sera contaminated with different concentrations of leptospires, while specificity was established using sera contaminated with different bacterial genera and sera obtained from patients positive for viral infections. IC-PCR using AS was able to recognize specific serogroups, although some cross-reactions have been observed. No cross-reactions were observed when MAbs were used; however, the sensitivity in this case was lower than that of IC-PCR using AS. IC-PCR proved to be specific to Leptospira and is a promising tool for early diagnosis of leptospirosis, providing additional information about the infecting serovar or serogroup.

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1. Introduction

Leptospirosis is recognized as an important zoonotic disease widespread worldwide, being most common in tropical regions (Levett, 2001; Ko et al., 2009). The infection is considered a public health problem in many countries, including Brazil, where most reports are focused on urban slums, especially due to exposure to rainwater contaminated with the urine of infected animals (Ko et al., 1999). The tropical climate, which provides periods of intense rainfall, resulting in floods, combined with a dense population living in poverty and poor sanitation conditions makes this country susceptible to the maintenance and spread of leptospirosis (Ko et al., 1999; Fonseca et al., 2006b).

The disease varies from a subclinical infection to a severe illness with multi-organ involvement, leading to fatal forms in some cases (Mérien et al., 1995; Levett, 2001). Early diagnosis is important and complicated owing to the wide spectrum of clinical symptoms of the disease, and the infection is frequently misdiagnosed as influenza, hepatitis, hemorrhagic fever, or dengue fever. Therefore, diagnosis should be based primarily on laboratory tests rather than on clinical aspects by themselves (Mérien et al., 1992; Branger et al., 2005).

The gold standard methodology for leptospirosis diagnosis, the microscopic agglutination test (MAT), is based on the antibody response of the host, which can occur only in a period of 8–10 days after the onset of symptoms, named seroconversion (Mérien et al., 1995). MAT presents the inconvenience of being laborious and requires the maintenance of living cultures (Fonseca et al., 2006a; Perez and Goarant, 2010). The diagnosis can also be based on the cultivation of leptospires, using blood collected from patients in the acute phase of the disease. However, the bacterial culture presents low sensitivity, about 30%, and may be a retrospective diagnosis, since it can take up to 2 months (Mérien et al., 1995; Fonseca et al., 2006a). Nevertheless, the isolation of Leptospira is extremely valuable, especially in epidemiologic studies, since it is possible to identify the infective serogroup/serovar.

Polymerase chain reaction (PCR) has been successfully used for the amplification of different DNA sequences of leptospires (Grave-kamp et al., 1993; Levett et al., 2005; Kositantorn et al., 2007; Slack et al., 2007; Bomfim et al., 2008) and is considered an important technique for the early detection of the microorganism, while other methods either failed or proved to be unreliable (Brown et al., 1995; Ooteman et al., 2006a). PCR is an extraordinarily useful tool for the detection of infectious agents that are difficult to cultivate, such as leptospires; however, it often restricts data available for epidemiologic surveillance, especially concerning the infective strain (Perez and Goarant, 2010).
Immunocapture-PCR (IC-PCR) has already been described as a rapid and efficient tool for detection and identification of *Salmonella*, *Shigella*, and *Mycobacterium* species (Luo et al., 2002; Peng et al., 2002; Warren et al., 2007; Chui et al., 2010; Katsuda et al., 2010). Generally, this assay is based on an initial step of concentration and immunologic capture of the pathogen, and a second step in which the microorganism is identified following the amplification of specific regions of its DNA. Even though this method has not been described for detecting *Leptospira* yet, it seemed to be a promising possibility. The main goal of this study was to develop a new IC-PCR that would combine the early diagnosis with the presumptive identification of *Leptospira* at the serovar or serogroup level.

2. Materials and methods

2.1. Bacterial strains and growth conditions

*Leptospira interrogans* serovars Icterohaemorrhagiae (RGA strain—reference no. CLEP 0001), Copenhageni (M20 strain—reference no. CLEP 0002), and Canicola (Hond Utrecht IV strain—reference no. CLEP 0003) were obtained from the Collection of *Leptospira* (CLEP, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil) and grown in Ellinghausen-McCullough-Johnson-Harris medium (Difco, Isère, France) at 28 °C. *Escherichia coli*, *Salmonella enterica*, *Shigella sonae*, and *Yersinia enterocolitica* were kindly provided by the Collection of Bacteria of Importance to Health (Oswaldo Cruz Foundation) and grown in brain heart infusion medium (Difco) at 37 °C.

2.2. Sera samples

Blood from human healthy donors was collected to provide serum. Twenty-nine sera samples were collected from patients diagnosed with a disease usually misdiagnosed as leptospirosis, such as dengue fever, hantaviruses, and viral hepatitis. All samples were provided by Dr. Marluce Aparecida Assunção Oliveira from the Fundação Ezequiel Dias (Minas Gerais, Brazil).

2.3. IC-PCR: Standardization for serogroup identification

Twenty-one rabbit antisera containing polyclonal antibodies against reference strains (Table 1), obtained from the Royal Tropical Institute (Amsterdam, The Netherlands), were separately coupled to polystyrene 96-well plates at 4 °C for 18 h. Sera were diluted 1:100 with 0.05 mol/L carbonate buffer (pH 9.6). The wells were washed with a solution (0.2 mol/L phosphate-buffered saline; 0.05% Tween 20; pH 7.6) and subsequently incubated with a block solution (0.2 mol/L phosphate-buffered saline; 2% bovine serum albumin; pH 7.6) at 37 °C for 1 h. The wells were washed again with the same buffer and incubated at 37 °C for 1 h with 100 μL of sera from healthy donors and artificially contaminated with 10^7 leptospires/mL. *L. interrogans* serovar Copenhageni M20 strain was used in this step. Sera without bacteria were used for the control wells. Following washing with 0.2 mol/L phosphate-buffered saline (pH 7.6), the wells were incubated with sterile Milli-Q water and heated at 100 °C for 10 min to obtain the bacterial DNA. The PCR mixture consisted of 10 mmol/L Tris–HCl buffer (pH 8.3) (Sinapse Biotecnologia, São Paulo, Brazil), 2 mmol/L MgCl2 (Sinapse Biotecnologia), 200 μmol/L of each deoxyribonucleotide (Promega, São Paulo, Brazil), 2 U of Taq DNA polymerase (Sinapse Biotecnologia), 1 μmol/L of fluB (Kawabata et al., 2001) and lip41 (Ahmed et al., 2006) primer pairs, and 5 μL of the DNA template obtained in the previous step. PCR was performed in a thermocycler (Gene Amp PCR System 9700, PE Applied Biosystems, Carlsbad, CA, USA), and the first amplification cycle consisted of denaturation at 95 °C for 5 min, 35 cycles of 95 °C for 30 s (denaturation), 54 °C for 30 s (annealing) and 72 °C for 1 min (extension), and a final extension at 72 °C for 7 min. Positive (PCR mix with DNA of *L. interrogans* serovar Copenhageni M20 strain) and negative (PCR mix without DNA) controls were also included in the reactions. The amplification products (10 μL) were mixed with 1 μL Blue Green Loading Dye (LGC Biotecnologia, São Paulo, Brazil) and subjected to electrophoresis on 1.5% agarose gel prepared with TAE (2 mol/L Trizma base; Sigma, St. Louis, MO, USA; 1.2 mol/L acetic acid; 0.5 mol/L ethylenediaminetetraacetic acid) buffer. The molecular size marker 100–bp DNA ladder (Sinapse Biotecnologia) was also included. The gels were analyzed under UV light.

2.4. IC-PCR for serovar identification

The procedure described above was used to couple monoclonal antibodies (MAbs) to polystyrene 96-well plates. In this step, specific MAbs against serovars *Icterohaemorrhagiae* (F70C14), *Copenhageni* (F70C24), and serogroup *Icterohaemorrhagiae* serovar non-icterohaemorrhagiae (F89C12) were used. Sera samples were artificially contaminated with 10⁷ cells/mL with the following reference strains: *L. interrogans* serovars Copenhageni (M20 strain—the reference no. CLEP 0002), *Icterohaemorrhagiae* (RGA strain—the reference no. CLEP 0001), and Canicola (Hond Utrecht VI strain—the reference no. CLEP 0003).

2.5. Sensitivity of IC-PCR

The sensitivity of IC-PCR was established by using sera artificially contaminated with increasing concentrations of *L. interrogans* serovar Copenhageni M20 strain (10⁷ to 10⁷ cells/mL), in plates coated with

Table 1

<table>
<thead>
<tr>
<th>Antiserum*</th>
<th>Serogroup</th>
<th>Serovar</th>
<th>Reference strain</th>
</tr>
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<tbody>
<tr>
<td>AS-1</td>
<td>Icterohaemorrhagiae</td>
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<td>M20</td>
</tr>
<tr>
<td>AS-2</td>
<td>Icterohaemorrhagiae</td>
<td>Icterohaemorrhagiae</td>
<td>RGA</td>
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<tr>
<td>AS-3</td>
<td>Canicola</td>
<td>Canicola</td>
<td>Hond Utrecht IV</td>
</tr>
<tr>
<td>AS-4</td>
<td>Grippotyphosa</td>
<td>Grippotyphosa</td>
<td>Moskva V</td>
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<td>Pomona</td>
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<td>Javanica</td>
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<tr>
<td>AS-25</td>
<td>Ballum</td>
<td>Kenya</td>
<td>Njenga</td>
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</tbody>
</table>

* Rabbit antisera were obtained from the Royal Tropical Institute–KIT Biomedical Research (Amsterdam, The Netherlands).

![Fig. 1. IC-PCR protocol standardization using reference antisera (AS), showing specific recognition of leptospires by polyclonal antibodies of serogroup Icterohaemorrhagiae and cross-reactions with heterologous serogroups represented by PCR products of lower intensity. Amplification reactions were disposed on the gel in order to compare specific control and test wells of each AS. M, 100-bp DNA ladder. A) a, Positive control for the PCR; b, negative control for the PCR; c/d, AS-1; e/f, AS-2; g/h, AS-3; i/j, AS-4; k/l, AS-5; m/n, AS-6; o/p, AS-10; q/r, AS-11; s/t, AS-12; u/v, AS-13; w/a (B), AS-14. B) b/c, AS-15; d/e, AS-16; f/g, AS-17; h/i, AS-19; j/k, AS-20; l/m, AS-21; n/o, AS-22; p/q, AS-23; r/s, AS-24; t/u, AS-25.
Icterohaemorrhagiae (data not shown).

with the plates coupled with MAbs of serovars Copenhageni and coated with MAbs of homologous serovars, and cross-reactions were determined. The results showed that PCR products were detected only in the wells artifically contaminated with serovars Icterohaemorrhagiae (Fig. 2B), and Canicola (data not shown), separately.

3.3. IC-PCR is sensitive and specific

Wells were coupled with AS-1 and MAb F70C24, separately, and the test was performed with sera artificially contaminated with a crescent of leptospires. The results showed that IC-PCR with reference antisera was 10 times more sensitive (limit of detection: 10^5 leptospires/mL; Fig. 3) than with monoclonal antibodies (limit of detection: 10^6 leptospires/mL; Fig. 4).

In order to compare IC-PCR and conventional PCR sensitivities, thermal lysis was applied directly to sera artificially contaminated with crescent concentrations of leptospires. In this case, the limit of detection by PCR was 10^7 leptospires/mL (data not shown).

In order to test the specificity of the protocol, wells were coupled with the same antisera and monoclonal antibodies used in the standardization step. The test was performed with sera artificially contaminated with other Gram-negative bacterial species presenting flagella and with sera obtained from patients previously confirmed for viral infections that are commonly misdiagnosed as leptospirosis. Any cross-reactions were detected in this step, confirming that the IC-PCR protocol standardized by our group is specific to Leptospira (data not shown).

4. Discussion

Leptospirosis is widespread around the world, being particularly common in tropical and subtropical regions where environmental conditions favor the survival and transmission of leptospires. The World Health Organization considers leptospirosis a neglected tropical disease and estimates the median global incidence of this infection to be at least 5.1 cases per 100,000/year in endemic areas and 14 cases per 100,000/year during epidemics (Lau et al., 2012).

Early detection of leptospirosis demands rapid and sensitive diagnostic tests (Fonseca et al., 2006b), and, usually, PCR-based approaches are the methods of choice for this purpose. A single positive sample provides a true result before seroconversion; however, this can lead to the loss of serology-based identification of the infective serovar, which hampers epidemiologic studies (Perez and Goarant, 2010).

In order to address this problem, Perez and Goarant (2010) proposed a direct multilocus sequence typing (MLST) scheme from clinical samples. Leptospira could be identified in sera using real-time PCR, and, subsequently, MLST was conducted to identify the infective serovar. Our proposal, based on IC-PCR, follows the same line of reasoning; however, the identification step is based on serologic characteristics rather than on genotypic ones. Although the genotypic classification of leptospires has increased in recent years, the phenotypic characterization is still important, since the molecular classification is incompatible with the system of serogroups, which has been established for decades (Doungchawee et al., 2007).

The IC-PCR protocol established by our group allowed the specific detection of leptospires in serum samples, since no amplification products were observed when sera contaminated with other
microorganisms were used. This result reinforces the value of this methodology, especially because the symptoms of leptospirosis during the acute phase are almost indistinguishable from other bacterial and viral febrile infections (Srimanote et al., 2008).

The specificity of the IC–PCR protocol was also observed as regards the detection of particular serogroups and serovars. When sera artificially contaminated with serogroup icterohaemorrhagiae were tested on plates coupled with different reference antisera, the specific recognition of M20 strain by polyclonal antibodies of serogroup icterohaemorrhagiae occurred. However, as expected, cross-reactions were also found when plates were coupled with reference antisera of heterologous serogroups, corroborating previous observations (Levett, 2003; Doungchawee et al., 2007). This can be explained by the fact that polyclonal antibodies are able to recognize a wide range of epitopes found in different serogroups of Leptospira. Nevertheless, it is important to emphasize that cross-reactions occurred as low-intensity PCR products, being distinguishable from the products related to the specific serogroup recognition.

We also noticed the specific serovar identification when MAbs were coupled to the polystyrene plates, and, in this case, no cross-reactions were observed. It is well established that monoclonal antibodies enable epitope-specific recognition (Doungchawee et al., 2007).

In the acute phase of the disease, also known as leptospiaraemia, leptospires may reach 10^6–10^7 microorganisms/mL in the blood of the patient (Ko et al., 2009). For this reason, we chose 10^7 leptospires/mL as the ideal concentration to artificially contaminate the serum used in the test, since it could mimic real conditions. We noticed that the IC–PCR protocol for serogroup identification was 10 times more sensitive (limit of detection: 10^10 leptospires/mL) than the protocol standardized to serovar identification (limit of detection: 10^6 leptospires/mL). This suggests that perhaps polyclonal antibodies can recognize a higher diversity of epitopes than monoclonal antibodies, enabling the recognition of a greater quantity of leptospires in the immunologic step of the protocol.

When thermal lysis was applied directly to sera, the limit of detection by PCR was 10^5 leptospires/mL. The problem of sensitivity of PCR applied to the diagnosis of leptospirosis was previously discussed by Bal et al. (1994). They concluded that different DNA extraction methods directly affect reproducibility and sensitivity, especially due to the presence of different types of inhibitors. Based on this premise, we can assert that the present IC–PCR protocol needs improvements in order to minimize the loss of leptospires during the immunologic concentration or the DNA extraction steps.

Taken together, our results indicate that, although adjustments are necessary, we successfully standardized an IC–PCR protocol suitable for use as a new tool for the early diagnosis of leptospirosis, providing additional information about the infecting serovar or serogroup, which is crucial for epidemiologic surveillance. Our next step will be the use of clinical samples collected from patients in the early days of disease and presenting clinical symptoms of leptospirosis.

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