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# LEPTOSPIROSIS DIAGNOSIS USING NESTED-PCR

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## ABSTRACT

Leptospirosis is a worldwide sanitary problem. Its clinical signs resemble that of other diseases like Dengue and Flu, and it is difficult to distinguish between them. Currently available diagnostic methods shown low sensitivity and specificity. Efforts have been made to develop simpler, faster and more efficient diagnostic methods. The aim of this work was to evaluate and optimize a Nested-PCR method for diagnosis of leptospirosis. Primers were designed to amplify a 264 bp region within the LipL32 gene. The sensitivity and specificity of the assay was evaluated using seven saprophytic serovars and 35 pathogenic serovars. This technique showed to be very specific for pathogenic serovars, however it lacked sensitivity. In order to enhance the sensitivity, another primer pair was designed which amplify a 183 bp region within the 264 bp region in *lip*L32 gene, and used in a Nested-PCR assay. This approach was much more sensitive than traditional PCR.

**Key words:** Leptospirosis, PCR.

## INTRODUCTION

Leptospirosis is an anthropozoonosis, endemically spread worldwide, caused by bacteria of the *Leptospira* genus (2). From 1985 to 1993, 20.341 leptospirosis cases were notified in Brazil, with an 11% lethality (3). In 1994, 2.099 new cases were notified, with the same rate of lethality (1). Unfortunately, the number of notifications have increased during the last few years.

The areas of research in leptospirosis that have been given more attention are isolation and serovars characterization; increasing quality in data collection about disease incidence in Brazil; new vaccines; and development of new diagnostic methods. The aim of this work was to develop and standardize a diagnostic method based on PCR and Nested-PCR to achieve higher sensitivity and specificity, for human as well as animal leptospirosis diagnosis.

## MATERIALS AND METHODS

### DNA Extraction from Leptospira Cultures

DNA was obtained by diluting leptospira cultures from $10^{-1}$ to $10^{-8}$ in 1% salt solution. The number of bacterial cells present in each dilution was determined by counting them using a Petroff-Houser chamber. Dilutions were centrifuged for 10 minutes, at 14000 rpm. The pellet was ressuspended in 50 µL of salt solution and boiled for 10 minutes. A volume of 10 µL was use in the PCR reaction.

### Primer Design and PCR Optimization

With the aim of developing a sensitive and specific assay, a primer pair was designed using Vector NTI 5.0 software (Informax Inc) and the *Leptospira lip*L32 gene sequence obtained from the GenBank®. The best primer pair suggested by the software allowed the amplification of a 264 bp fragment. Seven saprophytic

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and 35 pathogenic serovars were assayed. DNA from different bacteria species was included as control. PCR was performed in 25 µL reaction volume containing 10 µL of template DNA, 2.5 µL of 10 x reaction buffer, 200 µM of dNTP, 20 pmol of each primer and 0.5 U of *Taq* DNA polimerase (Invitrogen). Amplification was carried out in a Perkin-Elmer thermocycler using 35 cycles of 94ºC for 1 min, 55ºC for 1 min, and 72ºC also for 1 min.

**Nested-PCR Optimization**

In an attempt to increase sensitivity, another primer pair was designed, which amplifies a 183 bp region within the 264 bp sequence from *lip*L*32* gene. The template DNA used was 1 µL of the first PCR reaction. The program, reagents and volumes were the same as used for the first PCR. Clinical samples from infected animals were submitted to this test. These samples were obtained from Zoonosis Control Center (CCZ), UFPel. A volume of 10 µL of the amplification reaction was applied to a 2% agarose gel, stained with Ethidium Bromide, and visualized under UV.

## RESULTS

**Leptospira DNA Extraction**

DNA extraction protocol based on boiling bacterial cells suspended in 1% salt solution was simple and a good quality DNA was obtained. This method is cheap and effective in DNA extraction from leptospira cultures as well as from clinical samples.

**Primer Design and PCR Optimization**

The PCR sensitivity and specificity was evaluated using 7 saprophytic and 35 pathogenic serovars. A 264 bp fragment was obtained with an annealing temperature of 55ºC. Amplification was only observed from pathogenic serovars, but sensitivity was low, detecting a minimum of around 400 bacterial cells per mL.

**Nested-PCR Optimization**

Nested-PCR was carried out using a primer pair, which amplifies a 183 bp region within the 264 by of the first amplification. Sensitivity was greatly enhanced with this approach, allowing amplification from samples containing as few as 20 bacterial cells per mL.

Eighteen clinical samples from animals suspected of having leptospirosis were submitted to this test. Only one of them resulted in a visible band after the first PCR reaction, while 17 out of 18 samples tested were positive in the Nested-PCR (Fig. 1)

## DISCUSSION

We have described the development of a new Nested-PCR method for diagnosis of leptospirosis. This approach has the advantage of allowing detection of the pathogen soon after infection, unlike most methods currently used, which are based on detection of antibodies against the pathogens.

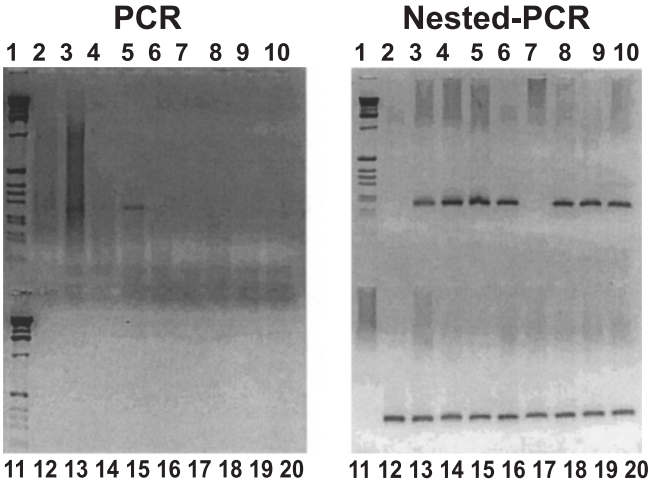


**Figure 1:** 2% agarose gel electrophoresis stained with Ethidium Bromide. **PCR:** columns 1 and 11, molecular weight marker Ladder 1 kb (Invitrogen); column 2, negative control; column 3, positive control; columns 4 to 20, suspected clinical samples from CCZ, UFPel. **Nested-PCR:** column 1, molecular weight marker Ladder 1 kb; columns 2 and 11, negative control; column 3, positive control; columns 4 to 20, suspected clinical samples from CCZ, UFPel.

The choice of target for the PCR was appropriate as the *lip*L32 gene is absent from non pathogenic leptospira or any other bacteria tested, resulting in a very specific test. The sensitivity problem detected after the first PCR reaction was overcome by the Nested-PCR reaction, which allowed an increase in sensitivity of at least 20 fold. The only drawback of this approach is the high risk of contamination by the amplicon, resulting in false positive results.

## RESUMO

**Diagnóstico de leptospirose utilizando Nested-PCR**

A leptospirose constitui um problema sanitário de importância mundial. Esta doença caracteriza-se por apresentar sintomas muito parecidos com os de outras doenças como a dengue e a gripe e, clinicamente é difícil de distingui-las. Técnicas de diagnóstico da leptospirose atualmente disponíveis apresentam baixa sensibilidade e ou especificidade. Por isso, tem havido um grande esforço no sentido de desenvolver testes rápidos e eficientes, baseados em técnicas de biologia molecular. Este trabalho objetivou a avaliação e otimização do Nested-PCR, para o diagnóstico de leptospirose. Para isso foi desenhado um par de primers que amplifica uma região de 264 pb do gene

LipL32. A sensibilidade e especificidade do teste foram avaliadas utilizando 7 sorovares saprófitas e 35 sorovares patogênicos. Este PCR mostrou-se específico para leptospiras patogênicas, porém a sensibilidade não foi muito alta. Com o objetivo de melhorar a sensibilidade do teste , foi desenhado outro par de primers que amplifica uma região de 183 pb do gene LipL32, interna a de 264 pb para a realização do Nested-PCR. Nesta reação foi utilizado como DNA molde o produto da primeira amplificação. O Nested-PCR mostrou-se mais sensível que o PCR normal, pois foi capaz de detectar um baixo número de células bacterianas.

**Palavras-chave:** Leptospirose, PCR.

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