

## Recommendations for the detection of *Leptospira* in urine by PCR

### Recomendações para detecção de *Leptospira* em urina pela PCR

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#### ABSTRACT

*In the present study PCR was applied to detect leptospires in human urine. Several approaches for sample processing were evaluated to optimize the detection of leptospires in urine mixed with this bacterium. Furthermore, some changes in the composition of the reaction mix were studied. No amplification was observed in acidic urine, therefore neutralization of the sample immediately after collection is strongly recommended. PBS gave better results than Tris or NaOH as neutralizing reagents. Freezing and thawing of samples before processing yielded negative results. Elimination of epithelial cells, leukocytes and crystals by centrifugation at 3,000 rpm at room temperature increased sensitivity. In addition, both the washing step after collecting leptospires by centrifugation and the inclusion of 0.1% bovine serum albumin in the reaction mix minimized the interference of other inhibitory compounds. These modifications were useful to improve the detection of *Leptospira* in urine by PCR.*

**Key-words:** *Leptospira. PCR. Urine. PCR inhibitors. Human.*

#### RESUMO

*No presente estudo, a PCR foi utilizada para detectar leptospiros em urina humana. Diversas abordagens para processamento de amostra foram avaliadas para otimizar a detecção de leptospiros em urina misturada com esta bactéria. Além disso, algumas mudanças na composição da mistura de reação foram analisadas. Não se observou amplificação em urina ácida, conseqüentemente, a neutralização da amostra imediatamente após a coleta é fortemente recomendada. PBS apresentou melhores resultados que Tris ou NaOH como reagentes neutralizadores. Congelamento e descongelamento de amostras antes do processamento produziram resultados negativos. Eliminação de células epiteliais, leucócitos e cristais por centrifugação a 3.000rpm, à temperatura ambiente, aumentou a sensibilidade. Ademais, ambas, a etapa de lavagem após a coleta de leptospiros por centrifugação e a inclusão de albumina de soro bovino a 0,1% na mistura de reação minimizaram a interferência de outros compostos inibidores. Essas modificações contribuíram para melhorar a detecção de *Leptospira* em urina através da PCR.*

**Palavras-chaves:** *Leptospira. PCR. Urina. Inibidores PCR. Humana.*

Leptospirosis is a widespread disease that affects wild and domestic animals as well as humans<sup>1,2</sup>. There are several reports on the frequency of leptospirosis among livestock, wild animals and human beings in South America mainly in Argentina and Brazil<sup>4,19,20</sup>. Humans may be infected indirectly from animals by contact with contaminated water, soil or mud in a moist environment, or by direct infection from urine, fresh carcasses or organs<sup>6</sup>.

There are many possible clinical presentations and courses of human leptospirosis. In the past two decades, an increasing

number of cases of leptospiral pulmonary hemorrhages have been reported<sup>8,19,21</sup>. As a consequence of the broad spectrum of nonspecific symptoms, leptospirosis can be misdiagnosed and clinical differential diagnosis is required between leptospirosis and severe influenza, viral meningitis, hepatitis, hemorrhagic fevers or nephritis, among other conditions<sup>6</sup>. A conclusive diagnosis cannot be made without laboratory confirmation.

Most cases of leptospirosis are diagnosed by serology and the reference method is the microscopic agglutination test (MAT)

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although it is a complex test to control, perform and interpret<sup>12</sup>. Live cultures of all serovars required for use as antigens must be maintained. Another drawback is that antibodies are only detectable in blood approximately 5 to 7 days after the onset of symptoms.

Efforts for early diagnosis of leptospirosis are directed towards the detection of leptospires or their DNA or antigens in blood, cerebrospinal fluid, urine, and tissues. The techniques available are direct examination for leptospires, culture, detection of leptospiral antigens with antibodies, and detection of leptospiral DNA with homologous nucleic acid sequence probes, with or without amplification by polymerase chain reaction (PCR)<sup>6,16</sup>.

The PCR assay can be applied to selectively amplify specific DNA sequences by more than 10<sup>6</sup> fold<sup>17</sup>. Therefore, PCR is very useful for the rapid detection of organisms involved in acute infections. In fact, oligonucleotide primers have been developed by several research teams and applied for the specific detection of certain serovars of *Leptospira*<sup>18, 22, 23</sup>. Gravekamp *et al*<sup>9</sup> designed two pairs of primers for the specific amplification of DNA of pathogenic leptospires. With some modifications, this method was applied in the analysis of several strains isolated in Argentina, from water, soil and human patients<sup>15</sup>.

Because endogenous substances present in urine can inhibit PCR, we report several approaches for sample processing and some changes in the composition of the reaction mix to optimize detection of this bacterium.

## MATERIALS AND METHODS

**Bacterial strain and culture medium.** *Leptospira interrogans* serovar *pomona*, donated by Dr. Gleyre Dorta de Mazzonelli (DILACOT-SENASA, Buenos Aires), was grown in EMJH medium<sup>5,11</sup> at 27°C and periodically subcultured in fresh medium. This strain had been typed by the cross-agglutination absorption test<sup>24</sup>.

**Urine mixed with leptospires.** Aliquots were taken from a *L. interrogans* serovar *pomona* culture in EMJH. Cells were washed twice in sterile distilled water by centrifugation at 12,000 rpm (microcentrifuge Sorvall RMC 14) for 20 min and resuspended in distilled water. After estimating the bacterial concentration by nephelometry, different aliquots were added to urine from a healthy human to achieve concentrations of 1,000, 10,000, 100,000 and 1 million bacteria per ml.

**Sample preparation for PCR.** Samples of artificially inoculated urine were incubated at 40°C for 10 min, to eliminate amorphous sediment, and then centrifuged at 3,000 rpm for 10 min at room temperature to eliminate epithelial cells, leukocytes and crystals commonly present in urine. Leptospires were concentrated by centrifugation at 12,000 rpm for 20 min, resuspended in 100 µl distilled water and boiled for 10 min. Two different volumes (10 and 35 µl) of sample were tested by PCR in a total volume of 50 µl.

To this general procedure the following modifications were made in order to increase the sensitivity of the PCR.

**Neutralization of urine:** urine was neutralized to pH 7.6 before adding the leptospires. Different solutions were used to achieve this pH: 0.1M Tris, 0.2M NaOH and phosphate-buffered saline (PBS).

**Freezing the urine:** samples of urine seeded with leptospires were subjected to freezing at -20°C, before centrifugation. This modification was analyzed to determine if urine samples could be frozen before processing.

**Washing the pellet of leptospires:** after centrifuging to concentrate leptospires and before boiling, a washing step with distilled water was added.

**Addition of bovine serum albumin (BSA) to the reaction mix:** different concentrations of BSA, from 0.013 to 0.1% (w/v), were added to the PCR reaction<sup>7</sup> instead of gelatin.

**PCR.** The reaction mix was constituted by 50mM KCl, 10mM Tris-HCl pH 9.0, 0.1% (v/v) Triton X-100, 2.5mM MgCl<sub>2</sub>, 0.01% (w/v) gelatin, 250µM each dNTP, 0.5µM each primer (G1, G2, B64-I, B64-II), 1 U *Taq* DNA polymerase. Amplification was performed with primers which only detect pathogenic leptospires<sup>9, 15</sup>.

One drop of mineral oil was added to each tube to prevent evaporation. PCR amplifications were performed as follows: one initial cycle of 94°C for 180", one final cycle of 72°C for 240" and 38 cycles of 90" at 94°C (denaturing), 90" at 55°C (annealing) and 150" at 72°C (extension).

A positive control with leptospires in the absence of urine was always run, and results were referred to this.

**Agarose gel electrophoresis.** Ten µl aliquots from each DNA amplification were analyzed by horizontal agarose gel electrophoresis and UV transillumination (300nm). Gel was constituted by 1.5% (m/v) agarose and 1.2g/ml<sup>-1</sup> ethidium bromide in running buffer (89 mM Tris, 89mM boric acid, 1mM EDTA, pH 8.0).

## RESULTS AND DISCUSSION

Sample processing for PCR is critical and must be adjusted to the tissue, fluid, and species being tested. Several substances found in the various types of clinical material inhibit PCR, therefore positive specimens may go undetected because of false-negative results.

There are many references about inhibition of *Taq* DNA polymerase by several factors such as chelation of free magnesium ions, hemoglobin, bile salts, acidic polysaccharides from glycoproteins and extreme pH variations<sup>3, 10, 13, 14</sup>. Phenol and chloroform, often used for DNA extraction and purification, are also considered to be inhibitors<sup>10</sup>.

As a consequence of the presence of inhibitors, some DNA purification steps are necessary before performing PCR amplification. These purification steps increase the cost of the test as well as the time required for diagnosis. Therefore, we tested procedures and conditions for sample preparation which are quick and inexpensive, and do not require the use of expensive kits to purify DNA.

Some bacteria can be lysed during the storage of the urine and, as a result, their DNA can be lost with the supernatant after centrifuging to concentrate the microorganisms. *Leptospira* is sensitive to acid, at pH 6.8 or lower<sup>6</sup>. In this study, after only a 90 min contact with acid urine, leptospires could not be detected

by PCR. This emphasizes the need to neutralize the urine sample immediately after collection, to avoid losing bacterial DNA in the washing step. PBS buffer rendered better results than either 1M Tris or 0,2M NaOH as neutralizing reagents (Figure 1, lanes 1 to 3).

When the urine mixed with leptospires was frozen at -20°C and thawed, before centrifugation steps, no leptospires could be detected by PCR (Figure 1, lanes 4 and 5). This suggests that when urine samples are going to be tested by PCR to detect leptospires, they have to be neutralized, and washed before storage at -20°C. In the case of samples that are not processed on the same day, they can be stored at 5°C until the following day, after neutralization.

The steps of sample preparation to improve the detection of leptospires in urine are summarized in Figure 2.

The optimum concentration of BSA used in the reaction mix for PCR was 0.1%. (Figure 3, lanes 13 to 15). This protein

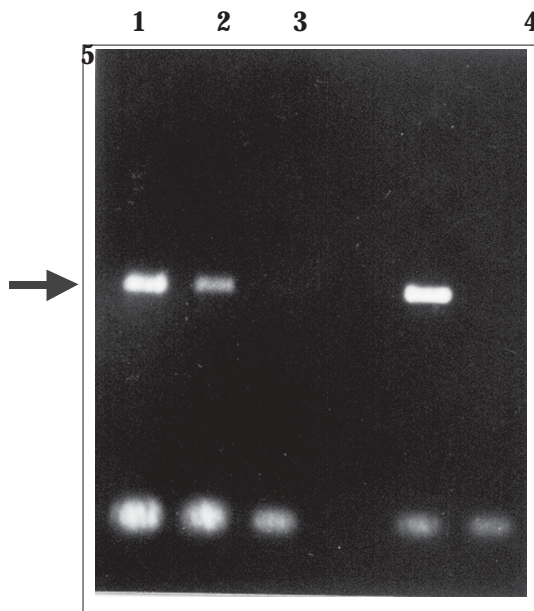


Figure 1 - Influence of the neutralizing agent on leptospiral DNA amplification from urine. Samples with: PBS (lane 1), 0.2 M NaOH (lane 2), 1M Tris (lane 3). Products corresponding to a sample frozen and thawed either after (lane 4) or before (lane 5) being processed for DNA extraction, were also electrophoresed.

plays a dual role since it can adsorb residual quantities of PCR inhibitors and also increases the stability in solution of *Taq* DNA polymerase.

A sample volume of 35µl in a 50µl PCR reaction mix rendered a band with a greater intensity than the use of 10µl of sample volume for PCR. However, there are cases in which increasing sample volume can result in increased inhibition of the enzyme, because inhibitory substances present in the sample also increase in quantity. The lower limit of detection was 10<sup>4</sup> leptospires in 1ml of urine.

The results obtained in this work are important to be considered for the implementation of the PCR assay to detect leptospires in clinical samples of human urine. It is important

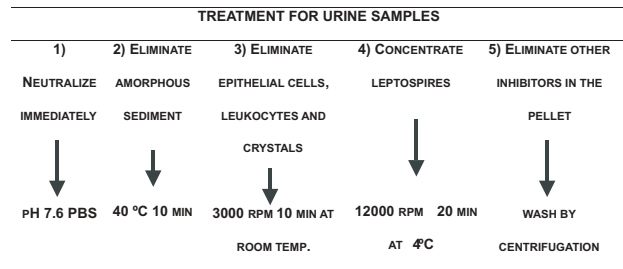


Figure 2 - Recommended steps for urine sample preparation.

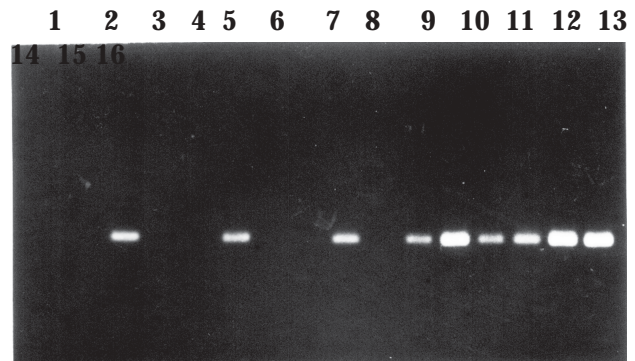


Figure 3 - Effect of bovine serum albumin (BSA) on the detection limit of leptospires in urine by PCR. Lanes 1, 2 and 3 correspond to urine containing 10<sup>8</sup>, 10<sup>6</sup> and 10<sup>4</sup> leptospires/ml, respectively, with 0.013% BSA added to the reaction mix; lanes 4, 5 and 6: with 0.025% BSA; lanes 7, 8 and 9: with 0.05% BSA; lanes 10, 11 and 12: with 0.078% BSA; lanes 13, 14 and 15: with 0.1% BSA; lane 16: positive control (amplification of urine-free leptospiral DNA).

to note that an internal control for the amplification reaction is necessary because inhibitory substances can vary among patients or between samples from the same patient.

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