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Comparison of Three Protocols to Preserve *Leptospira* spp. in Cat Urine for Efficient DNA Extraction and PCR Amplification*

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ABSTRACT

***Background*:** The pathogenic leptospira infection in mammalian species can cause a range of acute or chronic manifestations and may result in a carrier state. Previous studies have suggested that cats were resistant to acute leptospirosis however, the description of some clinical cases suggests that *Leptospira* spp. may also be pathogenic to this species. Recent studies have shown that leptospires may be shed in the urine of infected cats. Endogenous substances present in urine may inhibit PCR and allow leptospires to evade detection. This study aims to compare three protocols for sample processing to optimize the detection of pathogenic leptospires in cat urine.

***Materials, Methods & Results*:** Three protocols to optimize the detection of pathogenic leptospires in cat urine were tested. Aliquots of standard concentration of *L. interrogans* serovar Canicola culture were added to urine samples to achieve concentrations of 1×10^5 to 1×10^2 leptospires/mL for each protocol. In protocols A and B the urine was neutralized by the addition of phosphate-buffered saline (PBS), pH 7.4, in a proportion of 1 PBS: 2.5 urine (v/v). In protocol A, PBS was added to neutralize the urine pH for the leptospiral organisms immediately after addition of leptospires. In protocol B, PBS was added just before DNA extraction. In protocol C, no PBS was added. DNA extraction was performed at 4, 24 and 48 h after addition of the leptospires using a modified protocol. Samples were incubated at 37ºC for 10 min. Samples were then centrifuged (850 *g*) for 15 min, at 25ºC. The supernatants were transferred to another tube, and the pellets were discarded. The supernatants were centrifuged (16060 *g*) for 20 min at 4ºC. The supernatants were then discarded, and the pellets resuspended and washed with 1000 µL of PBS. All the samples were centrifuged at 16060 *g* for an additional 20 min at 25ºC. The supernatants were discarded and the pellets were resuspended in 100 µL of PBS and incubated at 94ºC for 10 min. DNA was stored at -20ºC until the molecular analysis. The PCR detection limit was evaluated. In samples from protocol A, leptospires were detected in concentrations up to 1×10^3 (4 h) and 1×10^4 (24 and 48 h). In protocol C, leptospires were detected in concentrations up to 1×10^4 (4 h) and 1×10^5 (24 and 48 h). No leptospiral DNA was detected in samples from protocol B.

***Discussion*:** Leptospires are sensitive to acid conditions, at pH 6.8 or lower and the urine pH of cats may vary from 5 to 7. In the present study, we found best results for DNA amplification with the addition of PBS immediately after urine collection (protocol A). Previous studies have shown the importance of neutralizing urine samples immediately after collection to avoid loss of bacterial DNA during the extraction process. However, protocols B and C may be an alternative in clinical practice, when PBS cannot be added immediately after collection. The delay after urine collection before DNA extraction is one more factor that may interfere with the PCR sensitivity. This was observed in the samples from protocol A, because although these samples were neutralized immediately, there was a 10-fold decrease in the detection limit of the test at 24 and 48 h. Leptospires rapidly lose their integrity in urine and the detection limit declines considerably over time, so prompt extraction is essential. These results show that the in-house method of preserving cat urine is useful to maintain the viability of leptospiral DNA extraction. In addition, this study highlights the importance of neutralizing urine samples immediately after collection and the need for prompt DNA extraction to improve PCR detection limit. However, if PBS cannot be added to the collected sample immediately, it is better to process the sample without PBS and extract DNA as soon as possible to minimize the risk of false-negative results.

Keywords: feline, leptospires, leptospirosis, molecular diagnostic.

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INTRODUCTION

Leptospirosis is a zoonotic disease caused by pathogenic leptospira species. The infection in mammalian species can cause a range of acute or chronic manifestations and may result in a carrier state for which the duration varies considerably between species [5]. Previous studies in cats have suggested that they are resistant to acute leptospirosis; however, the description of some clinical cases suggests that *Leptospira* spp. may also be pathogenic to this species [1-3,9,11,12]. Cats may shed leptospires intermittently in their urine for several weeks after experimental or natural infection [4,6,8,12,13]. These findings suggest that cats are a potential source of infection for human beings and others animals [12].

The diagnosis of leptospirosis in urine is performed by dark-field examination to identify leptospires or by PCR to detect leptospiral DNA. PCR is useful for rapid detection and provides high sensitivity, but sample processing has critical points and must be adjusted depending on the tissue, fluid, and species being tested [10]. Some DNA purification steps are also necessary before performing PCR amplification, because DNA degradation may lead to false negative results [10]. These steps increase the cost of the test because they require the use of expensive kits to purify DNA. In cats, some studies have been performed using commercial kits [4,6,12,13], but the extraction of DNA using in-house methods has not yet been reported.

The aim of this study is to compare three protocols for sample processing to optimize the detection of pathogenic leptospires in cat urine by in-house DNA extraction and further PCR testing.

MATERIALS AND METHODS

Bacterial strain

Leptospira interrogans serogroup Canicola, serovar Canicola, strain Hond Utrecht IV (CLEP 00003 FIOCRUZ) was used. The concentration of leptospires in a 2-week-old culture in liquid Ellinghausen-McCullough/Johnson-Harris medium was determined using a Petroff-Hauser Counting Chamber. The final concentration was 1×10^9 leptospires/mL.

Urine mixed with leptospires

Urine was collected by cystocentesis from a domestic shorthair cat with a strictly indoor lifestyle. The cat had 1-year-old, and no pre-existing illness nor was it receiving any medication. The urine pH was 6.

Aliquots of leptospire culture medium were added to aliquots of urine to achieve concentrations of 1×10^5 to 1×10^2 leptospires/mL for each protocol. Leptospires were added to all the samples at the same time (Figure 1).

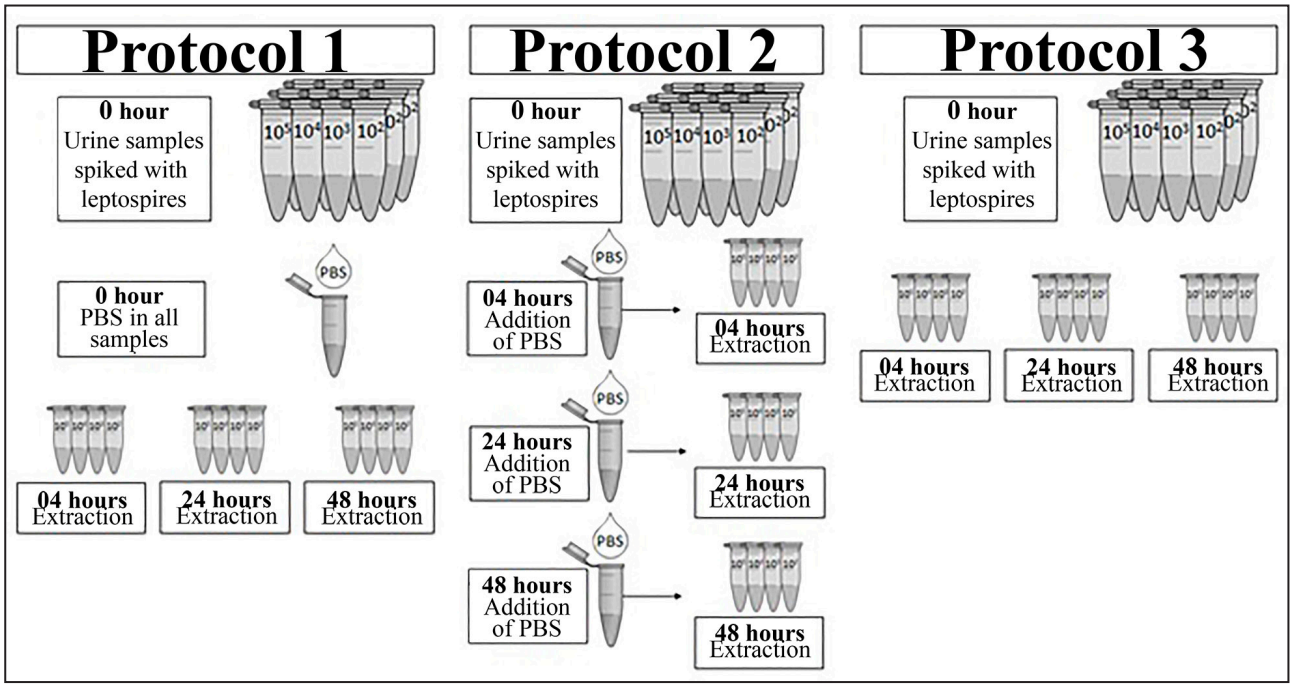

Figure 1. Demonstration of steps. Urine samples were spiked, neutralized and DNA was extracted according to three different protocols to compare the limits of PCR for detection of leptospiral DNA in cat urine.

Neutralization of urine

In all the three protocols the urine samples contained dilutions of 1×10^5 to 1×10^2 leptospires/mL. In protocols A and B the urine was neutralized by the addition of phosphate-buffered saline (PBS), pH 7.4, in a proportion of 1 PBS: 2.5 urine (v/v). In protocol A, PBS was added to the sample immediately after urine collection and addition of the leptospires. In protocol B, PBS was added just prior to DNA extraction. In protocol C, PBS was not added (Figure 1). All samples were refrigerated at 4ºC until DNA extraction was performed.

DNA extraction

DNA was extracted from urine samples at 4, 24 and 48 h using a modified protocol [10]. Samples were incubated at 37ºC for 10 min, to eliminate amorphous sediment. Samples were then centrifuged (850 *g*) for 15 min, at 25ºC. The supernatants were transferred to another tube, and the pellets were discarded to remove epithelial cells, leukocytes and crystals commonly present in urine. The supernatants were centrifuged (16060 *g*) for 20 min at 4ºC. The supernatants were then discarded, and the pellets resuspended and washed with 1000 µL of PBS. All the samples were centrifuged at 16060 *g* for an additional 20 min at 25ºC. The supernatants were discarded and the pellets were resuspended in 100 µL of PBS and incubated at 94ºC for 10 min. DNA was stored at -20ºC until the molecular analysis.

PCR

Previously described primers targeting the lipL32 gene (242 bp) of pathogenic leptospira species were used LIPL3245Fw (5′-AAG CAT TAC CGC TTG TGG TG-3′) and LIPL32286Rv (5′-GAA CTC CCA TTT CAG CGA TT-3′). PCR was performed in a total reaction mixture of 25 µL containing 5 µL of DNA template for the amplification, 1x PCR buffer1, 1.5 mM of $MgCl_2$, 0.2 µM of each desoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP), 1.0 U of Taq DNA polymerase (Recombinant® Taq DNA Polymerase)[2] and 0.2 µM of each primer.

PCR was performed using a thermocycler (SimpliAmpTM Thermal Cycler)[3]. The amplification protocol consisted of 3 min at 94°C for initial denaturation, 35 cycles of amplification (denaturation at 94°C for 45 s, annealing at 52°C for 45 s and extension at 72°C for 45 s and the final extension of 5 min at 72°C. Each run included a negative control (ultrapure water), DNA extraction negative control and a positive control (DNA extracted from leptospire cultures). The amplified PCR products were subjected to gel electrophoresis in 1.5% agarose gels for 1 h at 100V, followed by ethidium bromide staining (0.5 µg/mL TBE buffer). Visualization and photography of the bands of the expected size products were performed under UV light (L-PIX-HE®)[4] using the software L-PIX-IMAGE®[4].

The detection limit of the assay was measured by testing tenfold dilutions of fresh *L. interrogans* s. Canicola culture (10^8 to 10^1 leptospires/mL) in sterile PBS solution. DNA was further extracted and PCR performed.

RESULTS

The lower limit of detection was defined as the smallest number of organisms in a sample that could be detected by the PCR assay. Amplification of lesptospiral DNA was efficient up to 10^{-3} (5 copies/µL) dilutions of the starting template.

In protocol A, leptospiral DNA was detected in dilutions up to 1×10^3 when DNA was extracted at 4 h, and up to 1×10^4 at 24 and 48 h. In protocol B, no leptospiral DNA was detected. In protocol C, leptospires were detected in dilutions up to 1×10^4 when DNA was extracted at 4 h, and up to 1×10^5 at 24 and 48 h (Table 1).

Table 1. Detection limits of leptospiral DNA by PCR in cat urine comparing three protocols for sample storage.

Protocol	Hours for extraction	Dilution 1×10^5	Dilution 1×10^4	Dilution 1×10^3	Dilution 1×10^2
A	4	+	+	+	-
	24	+	+	-	-
	48	+	+	-	-
B	4	-	-	-	-
	24	-	-	-	-
	48	-	-	-	-
C	4	+	+	-	-
	24	+	-	-	-
	48	+	-	-	-

DISCUSSION

The PCR assay used here was able to detect 5 copies/μL of leptospiral DNA. Previous studies have reported detection limits of 4.15 copies/reaction in cats [12] and 50 copies/reaction in humans [10] both using G1 and G2 and B64-I/B64-II primers. The primer sets used in this study are not only highly sensitive but have another advantage; in contrast to PCRs using G1 and G2 and B64-I/B64-II primers, the pair of primers used in this study does not require a reaction multiplex or 2 single-reaction to include the genomospecie L. kirshneri, which contains serogroups commonly found in animals (Australis, Autumnalis, Bataviae, Canicola, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Pomona) [5].

Leptospires are sensitive to acid conditions, at pH 6.8 or lower [5] and the urine pH of cats may vary from 5 to 7 [7]. In the present study, we found best results for DNA amplification with the addition of PBS immediately after urine collection (protocol A). Previous studies have shown the importance of neutralizing urine samples immediately after collection to avoid loss of bacterial DNA during the extraction process [10]. However, protocols B and C may be an alternative in clinical practice, when PBS cannot be added immediately after collection.

However, when PBS was added immediately prior to extraction (protocol B), no leptospiral DNA was detected. This may be explained by two aggravating factors: the initial lack of neutralization plus the increase in the final dilution of the sample. During the detection limit assays, it was observed that after 4 h in contact with acid urine (pH 6), leptospiral DNA could not be detected by PCR in concentrations lower than 1×104 in the samples without PBS (protocol C). This indicates a 10-fold decrease in detection limit, compared to the samples of protocol A.

The delay after urine collection before DNA extraction is one more factor that may interfere with the PCR sensitivity. This was observed in the samples from protocol A, because although these samples were neutralized immediately, there was a 10-fold decrease in the detection limit of the test at 24 and 48 h. Leptospires rapidly lose their integrity in urine and the detection limit declines considerably over time, so prompt extraction is essential [10].

CONCLUSIONS

These results show that the in-house method of preserving cat urine is useful to maintain the viability of leptospiral DNA extraction. In addition, this study highlights the importance of neutralizing urine samples immediately after collection and the need for prompt DNA extraction to improve PCR detection limit. However, if PBS cannot be added to the collected sample immediately, it is better to process the sample without PBS and extract DNA as soon as possible to minimize the risk of false-negative results.

MANUFACTURERS

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***Ethical approval*.** This study was approved by the Ethics Committee on Animal Research from Universidade Federal do Parana (protocol number 057/2015).

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this manuscript.

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