Evaluation of polymerase chain reaction assay for pathogenic *Leptospira* detection in stored urine

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Abstract

Pathogenic *Leptospira* spp. is the etiological agent of leptospirosis, a worldwide zoonotic infectious disease that causes jaundice, hemorrhages, renal failure and abortion in susceptible species. Urine is one of the preferred clinical samples for the detection of the agent. However due to its reliability, detection of leptospires in stored samples is challenged. Here we evaluated the capability of a polymerase chain reaction (PCR) assay for detecting pathogenic leptospira DNA in a non-sterile collected urine, stored at different times and temperatures. Our results indicate that the PCR protocol used detect pathogenic leptospira DNA but not non-pathogenic serovars or other non-leptospiral microorganisms. The sensitivity of the assay was of 100 *Leptospira interrogans* in 10 mL refrigerated neutralized urine within 72 h post collection. This protocol could be of considerable interest for public health workers, field veterinarians and laboratory scientists, in sampling and processing urine for the detection of pathogenic *Leptospira* spp.

Introduction

Leptospirosis is a worldwide distributed zoonotic disease cause by pathogenic serovars of *Leptospira*. The agent infects susceptible hosts usually through mucous membranes or abraded skin.[1] After four to 10 day incubation period, leptospires disseminate throughout the body in a leptospiremic phase. With the appearance of circulating antibody, bacteraemia ceases and leptospires persist in immunoprotected sites, such as proximal convoluted tubules of the kidney.[2] Leptospires adhered to the tubular epithelial cells are excreted in urine for a variable period of time and contaminates the environment, where other susceptible animals are infect either directly or indirectly.

The main clinical signs of leptospirosis in animals are abortion, hemorrhages and jaundice, whereas liver failure, kidney damage, meningitis and respiratory distress are found in humans.[1] Clinical diagnostic of leptospirosis needs to be confirmed by laboratory tests. Serological tests (MAT, ELISA) demonstrate the presence of antibodies against leptospires in sera, which do not necessarily correlates with an active infection.[3,4] Usually, anti leptospiral antibodies appear one week after the infection, reach the peak two to three weeks later and then drop over weeks or months. A rising titer in successive specimens is then mandatory for serological diagnosis interpretation. Besides, it is not possible to determine the infecting serovar based exclusively on the results of serological tests. Technical methods to identify the presence of leptospires in clinical samples such as blood, urine, peritoneal or pleural exudates, or cerebrospinal fluid, are available, but most of them are laborious, biohazardous and poorly specific. For instances, direct observation under dark field microscope presents difficulties in obtaining suitable specimens and may provide false positive results due to its subjective interpretation in reading results.[5] Bacteriologic culture for leptospires is cumbersome, time consuming and usually restricted to highly specialized laboratories.[6] Isolation is always difficult due to the slow growth rate, particularly when combined with a concomitant contamination with faster growing microorganisms, and stringent and fastidious in vitro culture requirements of these bacteria. And the fluorescent antibody test, which is an excellent diagnostic technique, requires structural and antigenic integrity of the organism and technical skill to exclude confusing fluorescent elements.[7] Molecular techniques broadly improved the diagnosis of infectious disease. The polymerase chain reaction (PCR)-based strategy is a fast, accurate, widespread, specific and sensitive methodology that allows the detection of several microorganisms including leptospires, in a variety of specimens by amplification of a DNA fragment.[8] It requires a selection of specific primers to allow amplification of DNA segments common to all or specific to one strain.

Urine is one of the preferred clinical samples for detecting leptospiral infection.[2] Several PCR protocols have been published describing detection of leptospires in urine in humans[9-11] and animals[12-17] as well. However, all of these protocols were tested on urine samples collected or prepared in the laboratory under aseptic conditions and/or processed immediately after collection. Contrary to this, sometimes urine samples are collected and handled under non sterile conditions and frequently arriving to the laboratory for processing after 24 h post-collection. It is known that time and temperature of sample storage had pernicious effects for leptospira recovery,[18,19] in addition that urine contains deleterious components for leptospiral survival, DNA integrity and PCR assays.[5,11,12]

In this study, we evaluated the capability of a PCR assay to detect pathogenic *Leptospira* DNA in urine collected non-sterilely from the bladder and subjected to different incubation times and temperatures prior to analysis.

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Materials and Methods

Bacterial strains and culture conditions

Leptospiral strains used in this study were: *Leptospira (L.) interrogans* serovar (sv.) Canicola Hond Utrecht, sv. Copenhageni M20, sv. Pomona Pomona and sv. Hardjo Hardjoprajitno, and *L. biflexa*, sv. Patoc Patoc I. Other bacterial species used were *Escherichia coli*, *Brucella abortus* 2308, *Campylobacter fetus fetus*, *Corynebacterium bovis*, *Clostridium haemolyticum* and *Proteus mirabilis*. All bacteria were obtained from Instituto de Patobiología, Instituto Nacional de Tecnología

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Leptospires were propagated in Ellinghausen-McCullough-Johnson-Harris (EMJH) enriched medium at 28ºC and grown up for 5 to 7 days, when a uniform monolayer was observed under the dark microscope. Culture concentration was determined by direct counting in a Petroff-Hauser chamber. *E. coli*, *C. bovis* and *P. mirabilis* were plated onto blood agar plates and incubated at 37ºC for 24 h. *B. abortus* was cultured on tryptic soy agar (TSA) and incubated at 37ºC in an atmosphere containing 5% CO_2 for 4 days. *C. fetus fetus* was plated onto blood agar plate and incubated under microaerophilic conditions (10% CO_2, 5% O_2, 85% N_2) in an anaerobic jar at 37°C for 72 h. *C. haemolyticum* was cultured in 5% sheep blood agar and incubated at 37ºC for 48 hours under anaerobic conditions.

Urine sample collection and preparation

In order to reproduce a non-sterile urine collection, urine samples were collected in several occasions from one outdoor-subject cow serologically negative to leptospires (determined by MAT). Every time before collecting urine, the vulva was cleaned with tap water and dry with paper towel. After 30 seconds of perinea massage, a midstream sample of urine was collected and immediately taken to the lab.

The urine sample was mixed 50% (v/v) with sterile 2X phosphate-buffered saline (PBS), pH 7.6, and seeded with a pure culture of *L. pomona* to the final concentration of 1×10^7 leptospiras/mL of urine. Serial tenfold dilution were stored under 3 different conditions: Room temperature (20ºC), refrigerated (4ºC) and frozen (–20ºC). Room temperature and refrigerated samples were analyzed every 24 h for direct observation and DNA extraction. Frozen samples were frozen under slow process without any protecting agent, and they were sampled once after one-week storage.

Direct observation

Five µL of every dilution of spiked urine under each condition was evaluated and observed by dark field microscope at 100X. Ten ml of spiked urine was centrifuge at 10,000X g for 15 min at room temperature. Then, pellet was re-suspended in 1 mL of sterile distilled water, five µL placed in a slide and 10 fields were observed.

DNA extraction and polymerase chain reaction assay

Bacterial DNA was extracted using a commercial kit (QIAmp DNA mini kit, Qiagen, USA) following manufacturer instruction, and its concentration was measured by spectrophotometer (NanoDrop ND-1000, NanoDrop,

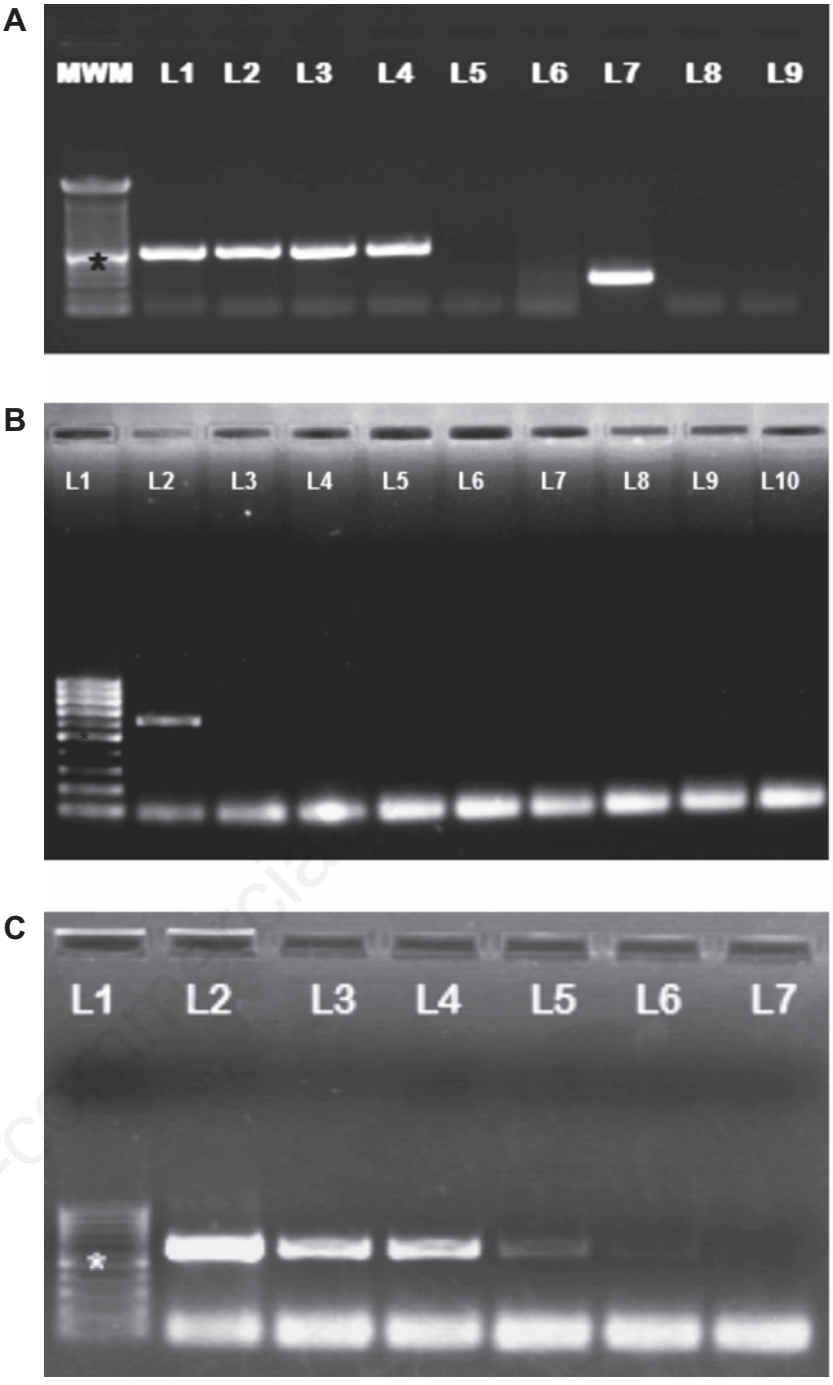

Figure 1. A) Agarose gel electrophoresis of PCR amplified products generated from leptospires DNA samples. L1 to L4 show 615 bp pathogenic leptospiras DNA amplification product using primer pair PU1-LepR1, while L7 shows 316 bp saprophytic leptospira DNA amplification product using primer pair SU1-LepR1. MWM: Molecular Weight Marker (100 bp DNA ladder; * indicates 500 bp); L1: *Leptospira* (*L.*) *interrogans* sv. Pomona (Pomona); L2: *L. interrogans* sv. Canicola (Hond Utrecht); L3: *L. interrogans* sv. Copenhageni (M20); L4: *L. interrogans* sv. Hardjo (Hardjoprajitno); L5: *L. biflexa* sv Patoc (Patoc I); L6: negative control; L7: *L. biflexa* sv Patoc (Patoc I); L8: *L. interrogans* sv. Pomona (Pomona); L9: negative control. B) Specificity test of primer set PU1 - LepR1. L1: DNA size marker (100 bp DNA ladder; * indicates 500 bp); L2: *L. interrogans sv.* Pomona (Pomona); L3: *Corynebacterium bovis*; L4: *Clostridium haemolyticum*; L5: *Campylobacter fetus fetus*; L6 *Proteus mirabilis*; L7: *Escherichia coli*; L8: *Brucella abortus* 2308; L9: *L.biflexa* sv. Patoc (Patoc I); L10: negative control. C) Sensitivity test of primer set PU1 – LepR1 using different concentration of genomic DNA from *L. interrogans* sv. Pomona Pomona. L1: DNA size marker (100 bp DNA ladder; * indicates 500 bp); L2-6: 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} leptospira. L7: negative control.

USA). The primers used in the PCR test were described by Kositanont *et al*.[20] and the master mix and PCR protocol was adapted to our laboratory conditions. Briefly, 38 µL of template from spiked urine or 10 ng of DNA extracted from pure bacterial cultures were subjected to PCR in a total volume of 50 µL reaction. The reaction mixture contained 1X buffer, 2 mM $MgCl_2$, 0.1 mM each of the four deoxynucleotide triphosphates (Invitrogen, USA), 0.4 µL of each primer (PU1, SU1 and LepR1);[20] and 1 U of mi-*Pfu* set DNA polymerase (Metabion, Germany). The mixtures were placed in an automatic PCR machine (Ivema T18, Argentina) and subjected to 40 2'50'' cycles. After an initial 5 min denaturation at 94°C, each cycle consisted in denaturation for 1 min at 94°C, annealing of primers for 50 sec at 50°C, and extension for 1 min at 72°C. A final cycle was identical, except that the extension step lasted 7 min instead of 1 min. PCR products were analyzed by gel electrophoresis. Ten ng of leptospiral DNA extracted from pure culture and reaction mixture without template (PBS, distilled water or urine) was used as positive and negative controls, respectively. Assays were repeated twice in duplicate.

Sensitivity and specificity of polymerase chain reaction assay

Specificity of PCR assay was tried using different strains of pathogen leptospires, one saprophytic leptospira strain and other non-leptospiral microorganisms which are etiological agents of bacterial diseases with clinical symptoms similar to leptospirosis. Pure cultures of leptospires (~10^8 leptospires/mL) were diluted 1:10 in distilled water, while colonies of grown bacteria in solid media were picked and suspended in 1 mL of distilled water to a cell density of 10^6/mL. Bacterial suspensions were centrifuged at 15,000 g for 10 min at 4°C and pellets re-suspended in 200 µL of 1X PBS previous to DNA extraction.

PCR sensitivity was evaluated in serial tenfold dilutions of leptospira free-bovine urine seeded with *L. pomona* cells. In order to eliminate amorphous sediments, 10 ml of seeded urines were initially centrifuged at 5000X g for 10 min at 4°C, after which the supernatant was transferred to a second sterile centrifuge tube. Following a centrifugation at 15,000X g for 15 min at 4°C, pellets were re-suspended in a 200 L of 1X PBS previous to DNA extraction.

Results

Direct observation

Detection of leptospires at T0 (immediately after processing) by direct observation under dark field microscope was only possible in samples containing 10^7 leptospires/mL. At later time points (T24, T48 and T72) leptospires were detected in refrigerated urine batch with 10^7 leptospires/mL, but not in refrigerated samples with lower leptospires concentration or batches kept at room temperature. No leptospires were observed in thawed samples. Altogether, these results indicate that 10^7 leptospires/mL is the detection limit for direct observation in fresh or refrigerated samples.

Specificity and sensitivity of polymerase chain reaction assay

The primer sets PU1-LepR1 and SU1-LepR1 showed to be specific for pathogenic and saprophytic *Leptospira* detection, respectively.[20] The PU1-LepR1 primer set amplified a product of 615 base pairs (bp) in all pathogenic *Leptospira* strains tested, but no amplification was observed when saprophytic *Leptospira* or other bacteria genus was employed (Figure 1A,B). On the contrary, the primer set SU1-LepR1 amplified a 316 bp saprophytic *Leptospira* DNA fragment. No amplification was observed when pathogenic *Leptospira* DNA was used as a template (Figure 1A).

The detection limit of the assay was 1×10^2 leptospira/mL (1 pg) determined by serial tenfold dilutions of *L. pomona* in water and urine (Figure 1C).

Polymerase chain reaction detection of pathogenic Leptospires in urine under different storage conditions

To evaluate the usefulness of the PCR to detect leptospira presence in non-sterile collected urine, we artificially inoculated a 10 mL leptospira free-bovine urine with *L. pomona* cells to a final concentration of 1×10^7 leptospires/mL of urine. Serial tenfold dilutions were maintained under room temperature (~20°C), refrigerated (4°C) or frozen (–20°C). Room temperature and refrigerated samples were sampled every 24 h, while frozen samples were thawed once after seven-day storage. In samples kept at room temperature, the sensitivity of the assay was 10^2 and 10^7 leptospires at 24 and 48 h post-inoculation, respectively. Leptospira DNA could not be detected in urine samples storage at room temperature for 72 h (Table 1). Adicionally, in refrigerated samples the limit of detection was 10^2 leptospires in all time points evaluated (from T24 through T72); while PCR detected up to 10^5 leptospires in once frozen-and-thawed samples. These results highlighted the importance of buffered and refrigerated urine samples immediately after its collection for PCR leptospira detection.

Table 1. Limit of *Leptospira interrogans* detection in urine by polymerase chain reaction under different storage condition. Numbers in the body of the table indicate leptospires concentration.

	T24	T48	T72
Room temperature	10^2	10^7	ND
Refrigerated	10^2	10^2	10^2

ND, no detected. T24, T48 and T72 = 24, 48 and 72 h post-inoculation.

Discussion

Urinary detection of leptospires is arduous because of the reliability of the agent and the intermittent and variable amount of leptospires spread with the urine.[1] Therefore many samples collection are recommended and two or more simultaneous diagnostic techniques should be used to maximize successful results. Direct observation by darkfield microscopy, bacteriologic culture with specialized culture medium and prolonged incubation times, immunofluorescence, and PCR are four widely available methodologies for leptospires detection in clinical samples.

Several PCR protocols to detect DNA of *Leptospira* spp. in different specimens (urine, blood, tissues) have been published, however few of them are able to differentiate DNA from pathogenic than saprophytic serovars. Among these protocols, the one described by Kositanont *et al*.[20] contains a primer set specific for amplifying a fragment of pathogenic *Leptospira* spp. DNA. Other protocols with similar abilities require two sets of primers,[21-23] separate tubes[24] or a combination of PCR with other molecular techniques,[14,25] which add additional time-consuming steps.

Interestingly, Kositanont *et al*.[20] had tested the sensitivity and specificity of the primer set in tissue and blood of rats naturally infected and human patients with suspected leptospirosis, respectively; but they did not evaluate the sensitivity and specificity of the assay in urine samples. Considering that urine is one of the preferred clinical samples for detecting leptospires in infected hosts, we developed a protocol including a primer set PU1-Lep1 for a quick diagnostic methodology of leptospirosis in urine. Branger *et al*.[16] also designed a

primer set that amplified DNA from pathogenic *Leptospira* spp. but not from other *Leptospira* serovars or other bacteria species; nevertheless it showed low sensitivity in frozen urine samples.

In this study, the limit detection of pathogenic leptospiral DNA in spiked urine was 100 cells/mL (Figure 1C). This result is in accordance with other PCR assays evaluated in urine samples, which detected from 10 to 200 leptospires/mL urine.[9,10,12-16] Considering that cows shed around 4×10^4 leptospires/mL urine during a peak of infection[13] and the agent is intermittently eliminated at variable amount in urine at later time points, the PCR sensitivity reported in this study is worthwhile. Our protocol showed similar detection limit of pathogenic leptospiral DNA when leptospira culture was diluted in water instead of urine. This result is different with other studies that showed higher sensitivity to detect leptospiral DNA in non-urine fluid than in urine,[9,16] which was attributed to the presence of some PCR-inhibiting factors in urine.[11,12]

The sensitivity of the assay was stable through time in refrigerated but not in room temperature storage samples (Table 1). Merien *et al*.[9] reported successful detection of *Leptospira* DNA in storaged samples, however different to this study, DNA was immediately extracted and PCR performed 48 h later. In other study, PCR assay detected *Leptospira* DNA in refrigerated dog urine after 48 h post-collection under sterile environment, but no sensitivity was tested.[17] This assay showed lower sensitivity in frozen than in refrigerated samples (10^5 *vs*. 10^2 leptospires), possible because freeze and thaw led to DNA degradation. This result is in concordance with Branger *et al*.[16] who detected only 30% of positive frozen samples. These data indicate that PCR must be performed immediately on fresh samples or in samples storage under refrigeration to increase the possibilities of positive results.

Leptospires die quickly in urine,[5] consequently better chances to preserve the agent are present when freshly voided specimens are processed immediately. In order to avoid or postpone detrimental effects of urine components on the agent, urine pH was neutralized and kept refrigerated until processed. Interestingly, viable leptospires were observed in buffered refrigerated spiked urine within 72 h post-process by direct observation. This result supports others[17,18] that show that delay recovery of leptospires from infected samples is possible when samples are stored at 4°C. Furthermore, no viable leptospires were observed from frozen samples. Freeze and thaw are deleterious for leptospires; perhaps the addition of a cryoprotective agent such as glycerol[26] may have minimized the deleterious effects of freezing. In addition, the results presented here and previously[20] shown that the set of primers used is specific for detecting pathogenic leptospires but not non-pathogenic leptospira or other bacteria in urine, tissues and blood.

In conclusion, this study shows a useful urine sampling and processing protocol for public health professionals, field veterinarians and laboratory workers for a quick, specific and sensitive detection of *Leptospira interrogans* DNA. This study is complementary to others,[18,20] and together allow a rapid diagnosis of leptospirosis through diverse clinical samples.

References

1. Faine S, Adler B, Bolin CA, Perolat P. Leptospira and leptospirosis. 2nd ed. Melbourne, Australia: MediSci; 1999.
2. Thiermann AB. Leptospirosis: current developments and trends. JAVMA 1984;184:722-5.
3. Thiermann AB. Bovine leptospirosis: bacteriologic versus serologic diagnosis of cows at slaughter. Am J Vet Res 1983;44:2244-5.
4. van den Broek AHM, Thrusfield MV, Dobbie GR, Ellis WA. A serological and bacteriological survey of leptospiral infection in dogs in Edinburgh and Glasgow. J Small Anim Pract 1991;32:118-24.
5. Alexander AD. Leptospira. In: Ballows A, Hausler WJ, Herrmann KL, et al, eds. Manual of clinical microbiology. Washington DC: American Society for Microbiology; 1991. pp 554-559.
6. Faine S. Guidelines for the control of leptospirosis. Geneva: World Health Organization; 1982.
7. Rossetti CA, Roge A, Bordagorria X, et al. Desarrollo y evaluación de un conjugado fluorescente para el diagnóstico de leptospirosis. Rev Med Vet (B Aires) 2009;90:17-21.
8. Saiki RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988;239:487-91.
9. Merien F, Amouriaux P, Perolat P, et al. Polymerase chain reaction for detection of Leptospira spp. in clinical samples. J Clin Microbiol 1992;30:2219-24.
10. Bal AE, Gravekamp C, Hartskeerl RA, et al. Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. J Clin Microbiol 1994;32:1894-8.
11. Lucchesi PM, Arroyo GH, Etcheverria AI, et al. Recommendations for the detection of Leptospira in urine by PCR. Rev Soc Bras Med Trop 2004;37:131-4.
12. Van Eys GJ, Gravekamp C, Gerritsen MJ, et al. Detection of leptospires in urine by polymerase chain reaction. J Clin Microbiol 1989;27:2258-62.
13. Gerritsen MJ, Olyhoek T, Smits MA, Bokhout BA. Sample preparation method for polymerase chain reaction-based semi-quantitative detection of Leptospira interrogans serovar hardjo subtype hardjobovis in bovine urine. J Clin Microbiol 1991;29:2805-8.
14. Wagenaar JA, Segers RP, Van der Zeijst BA. Rapid and specific detection of pathogenic Leptospira species by amplification of ribosomal sequences. Mol Biotechnol 1994;2:1-14.
15. Cai HY, Hornby G, Key DW, et al. Preliminary study on differentiation of Leptospira grippotyphosa and Leptospira sejroe from other common pathogenic leptospiral serovars in canine urine by polymerase chain reaction assay. J Vet Diagn Invest 2002;14:164-8.
16. Branger C, Blanchard B, Fillonneau C, et al. Polymerase chain reaction assay specific for pathogenic Leptospira based on the gene hap1 encoding the hemolysis-associated protein-1. FEMS Microbiol Lett 2005;243:437-45.
17. Harkin KR, Roshto YM, Sullivan JT, et al. Comparison of polymerase chain reaction assay, bacteriologic culture, and serologic testing in assessment of prevalence of urinary shedding of leptospires in dogs. J Am Vet Med Assoc 2003;222:1230-3.
18. Miller DA, Wilson MA, Beran GW. The effect of storage time on isolation of Leptospira interrogans from bovine kidneys. J Vet Diagn Invest 1990;2:63-5.
19. Fairbrother JM. Effects of products of autolysis of tissue and urine on the viability, morphology, and antigenicity of Leptospira interrogans serovar pomona. J Clin Microbiol 1985;21:189-94.
20. Kositanont U, Rugsasuk S, Leelaporn A, et al. Detection and differentiation between pathogenic and saprophytic Leptospira spp. by multiplex polymerase chain reaction. Diagn Microbiol Infect Dis 2007;57:117-22.
21. Gravekamp C, Van de Kemp H, Franzen M, et al. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. J Gen Microbiol 1993;139:1691-700.
22. Murgia R, Riquelme N, Baranton G, Cinco M. Oligonucleotides specific for pathogenic and saprophytic leptospira occurring in water. FEMS Microbiol Lett 1997;148:27-34.
23. Jouglard SD, Simionatto S, Seixas FK, et al. Nested polymerase chain reaction for detection of pathogenic leptospires. Can J Microbiol 2006;52:747-52.
24. Woo TH, Smythe LD, Symonds ML, et al. Rapid distinction between Leptospira

interrogans and Leptospira biflexa by PCR amplification of 23S ribosomal DNA. FEMS Microbiol Lett 1997;150:9-18.
25. Kawabata H, Dancel LA, Villanueva SY, et al. flaB-polymerase chain reaction (flaB-PCR) and its restriction fragment length polymorphism (RFLP) analysis are an efficient tool for detection and identification of Leptospira spp. Microbiol Immunol 2001;45:491-6.
26. Rossetti CA, Auteri CD. Long-term preservation of leptospiras by liquid nitrogen. Rev Argent Microbiol 2008;40:86-8.