

## Detection of Leptospire in Urine by PCR for Early Diagnosis of Leptospirosis

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**We tested urine samples from patients at different stages of current leptospirosis and thereafter to determine whether use of the PCR for detection of leptospire in urine can be a valuable alternative to culturing. The procedure of DNA extraction and subsequent PCR applied to 15 freshly voided urine samples proved to be twice as sensitive as culturing. Overall, we were able to detect leptospire in approximately 90% (26 of 29) of the urine samples. Urine and serum samples were obtained from seven patients, before the eighth day of illness. Although it is generally assumed that leptospiuria starts approximately in the second week of illness, we were able to detect leptospire in all of these early urine samples. In contrast, only two of seven corresponding serum samples gave positive PCR results, which suggests that PCR analysis of urine can be more successful for early diagnosis of leptospirosis than PCR analysis of serum. Urine samples from six patients who had been treated with antibiotics at the time of illness were positive by PCR, implying that the patients were still shedding leptospire in their urine despite treatment. Some of these samples were even taken years after the infection, indicating that shedding of leptospire in urine may last much longer than is generally assumed. We conclude that detection of leptospire in urine with PCR is a promising approach for early diagnosis of leptospirosis and may also be useful in studying long-term shedding.**

Diagnosis of leptospirosis is usually based on the demonstration of serum antibodies with serological tests like the microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA). In humans, antileptospiral antibodies become detectable at about the seventh day of illness. As a result of the immune response, leptospire are cleared from the blood after approximately the 10th day of illness. At this stage, some bacteria may remain in the convoluted tubules of the kidneys. It is generally assumed that shedding of leptospire in urine starts in the second week of illness and lasts 4 to 6 weeks (11), although rare cases of leptospiuria that lasted several months have been reported (12).

Besides serology, which becomes informative only after the seventh day of illness, culturing of leptospire from blood or urine can be used in the diagnosis of leptospirosis. Although culture medium can be inoculated immediately after onset of the disease, the results of culturing come late and therefore do not contribute to a rapid diagnosis. In addition, leptospire can be fastidious and often fail to grow in culture medium. Alternative methods developed to assess the presence of leptospire in clinical samples, like immunofluorescence staining (25, 32), immunoperoxidase staining (26), or DNA hybridization (28), were not satisfactory for routine diagnostic purposes, mainly because of their lack of sensitivity.

The PCR is a sensitive, specific, and rapid technique which has been successfully applied to the detection of several microorganisms and viruses in a variety of specimens, including sputum, serum, cerebrospinal fluid, urine, feces, and various tissues (4, 16, 19, 23, 30). Detection of leptospire in serum of

patients with leptospirosis by using PCR has recently been reported by Gravekamp et al. (10). Although PCR analysis of serum was more sensitive than culturing, a disappointing 50% was missed by PCR, possibly because the number of leptospire in these samples was too small to be detected by PCR. PCR analysis of urine may provide a good alternative to PCR analysis of serum, since in contrary to serum, urine samples can be collected very easily in large quantities and the sediments of large volumes of urine can be examined by PCR. Therefore, we think that exploration of PCR for the detection of leptospire in urine is worthwhile.

Detection of leptospire in urine by PCR has been reported for cattle (8, 29) and recently for one patient with leptospirosis (18). The use of PCR provides a considerable time gain compared with culturing of leptospire from urine, and PCR can be applied to frozen or formalin-preserved urine samples. In contrast, culturing is possible only with freshly voided specimens as leptospire die quickly in urine.

In this report, we describe the use of PCR to demonstrate leptospire in urine samples from patients with leptospirosis at different stages of the disease and thereafter. PCR results are compared with those obtained by culturing and by PCR analysis of serum samples to determine whether PCR analysis of urine can provide a serious alternative to methods currently used for diagnosis of leptospirosis.

### MATERIALS AND METHODS

**Clinical samples.** For PCR and culture, urine and blood samples were obtained between 1988 and 1993 from 29 patients in The Netherlands who were seropositive for leptospirosis. Twenty negative urine samples and 10 negative serum samples, used as controls, were obtained from healthy individuals or from patients with infections other than leptospirosis.

Of the 29 urine samples from patients with leptospirosis, 15 were freshly obtained and subjected to culturing and PCR. The

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other 14 urine samples, preserved with 0.1% formalin, were tested by PCR only.

Seven urine samples and corresponding blood samples were taken from patients who were ill for less than 8 days. Twenty-two samples were collected from patients at times varying from several weeks (2 to 14 weeks) to years (1 and 7 years for patients P2053 and P1885, respectively) after onset of the disease. Of these 22 patients, 6, including P1885 and P2053, were treated with antibiotics (penicillin, amoxicillin, and vibramycin) within the first 5 days of illness. Patient P1885 was additionally treated with doxycycline 3 years after onset of the disease. Treatments with antibiotics apparently were not adequate for patients P1885 and P2053, who suffered from persistent tiredness and headache. Immunoglobulin M titers against hardjo antigen in the corresponding sera of patients P1885 and P2053 were <1:20 (acute-phase titer not available) and 1:40 (1:1,280 in the acute phase), respectively.

To collect serum, blood was clotted at room temperature and subsequently centrifuged. Serum samples of 2 to 3 ml were collected, divided into 1-ml aliquots, and subsequently stored at  $-20^{\circ}\text{C}$  until DNA isolation.

The volumes of the urine samples that were obtained varied from 20 to 300 ml. Leptospire were collected from the complete samples by centrifugation for 10 min at  $3,000 \times g$ . After the supernatant was discarded, leptospire in the pellet were resuspended in 300  $\mu\text{l}$  of phosphate-buffered saline (pH 7.2) (22). In the case of freshly obtained urine, 150  $\mu\text{l}$  of this suspension was used for culturing. An equal volume of L6 buffer containing guanidinium thiocyanate (GuSCN; Fluka Chemie AG) (120 g of GuSCN, 22 ml of 0.2 M EDTA, 2.6 ml of Triton X-100 in 100 ml of 0.1 M Tris-HCl [pH 6.4]) was added to the remaining sample. These pretreated urine samples were divided into equal aliquots and stored at  $-20^{\circ}\text{C}$  until DNA isolation. The complete samples were tested in two separate PCR experiments; i.e., each aliquot of a sample was tested twice by PCR.

**Serology.** Several serum samples were collected from each patient during the acute phase of the disease and afterwards. The levels of antileptospiral antibodies in sera from patients were determined by MAT and ELISA. MAT and ELISA were applied as described earlier (5, 27). A battery of live leptospiral strains, as recommended by Faine (7), were used as antigens in the MAT. The ELISA was applied with heat-stable, broadly reactive antigen prepared from strains Wijnberg (serovar copenhageni) and Hardjoprajitno (serovar hardjo).

**Culture.** Leptospire were cultured in EMJH medium as described by Johnson and Harris (13). Inoculation of urine was performed within 2 h after voiding, in EMJH medium containing 200  $\mu\text{g}$  of 5-fluorouracil per ml to inhibit growth of contaminating microorganisms and in 5-fluorouracil-containing medium enriched with 1% (vol/vol) rabbit serum and 1% (vol/vol) fetal calf serum. The cultures were screened for leptospiral growth at regular intervals. A urine sample was considered negative if no growth was observed after 3 months.

**Identification of strains.** The strains isolated from human urine samples were typed by the cross-agglutination absorption test (5) and by use of monoclonal antibodies in agglutination tests (15).

**Isolation of DNA from clinical samples.** For isolation of DNA from clinical samples, the protocol described by Boom et al. (2, 3) was applied with minor modifications. Briefly, aliquots of pretreated urine samples or 1-ml samples of serum were mixed with 9 volumes of L6 buffer. Subsequently, 40  $\mu\text{l}$  of a diatom suspension (0.5 ml of 36% [wt/vol] HCl and 10 g of diatoms [Riedel-de Haën] in 50 ml of  $\text{H}_2\text{O}$ ) was added. After being vortexed thoroughly, the mixtures were left at room

temperature for 10 min, vortexed again, and centrifuged to collect the diatoms. The diatoms with absorbed DNA were subsequently washed twice with 1 ml of washing buffer (120 g of GuSCN in 100 ml of 0.1 M Tris-HCl [pH 6.4]), twice with 70% (vol/vol) ethanol, and once with acetone. After drying of the diatom pellets at  $56^{\circ}\text{C}$ , DNA was eluted in 125  $\mu\text{l}$  of  $\text{H}_2\text{O}$  for 10 min at  $56^{\circ}\text{C}$  in the presence of 200 ng of proteinase K per ml. To inactivate the proteinase K, samples were incubated at  $100^{\circ}\text{C}$  for 15 min.

The maximum initial volume of urine per aliquot that was processed for PCR was 50 ml. During introductory experiments, it was found that the sediment to be examined by PCR should not be obtained from more than 50 ml of urine to avoid interference with PCR by inhibiting substances. For the same reason, the volume of serum to be examined by PCR should not exceed 1 ml (10).

With each extraction procedure, several negative control samples consisting of distilled water, as well as negative urine and serum samples, were processed in parallel to monitor cross-contamination between samples.

**PCR.** We used two sets of primers for PCR, i.e., G1-G2 and B64-I-B64-II, as described previously (9, 10). With G1-G2, a 285-bp DNA fragment is amplified from leptospiral strains belonging to the pathogenic species *Leptospira interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. santarosai*, and *L. meyeri* (strain ICF only) (33). B64-I-B64-II amplifies DNA from leptospire belonging to *L. kirschneri* (21), generating a 563-bp DNA fragment. Primers were synthesized on an Applied Biosystems DNA synthesizer. A 40- $\mu\text{l}$  volume of each sample was used for amplification in a total volume of 50  $\mu\text{l}$ . Reactions were performed in Tris-HCl buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 3.0 mM  $\text{MgCl}_2$ ) containing each deoxynucleoside triphosphate (dATP, dTTP, dCTP, dGTP, and dUTP; Pharmacia LKB) at 250  $\mu\text{M}$  each primer at 1  $\mu\text{M}$ , 10  $\mu\text{g}$  of gelatin per ml, 0.5 U of *Taq* polymerase (Perkin-Elmer Cetus), and 0.5 U of uracil DNA glycosylase (BRL Life Technologies Inc.).

The reaction mixtures were covered with 50  $\mu\text{l}$  of mineral oil and incubated for 5 min at  $50^{\circ}\text{C}$  and 10 min at  $94^{\circ}\text{C}$  and then subjected to 34 cycles of denaturation for 1 min 30 s at  $94^{\circ}\text{C}$ , annealing for 1 min at  $55^{\circ}\text{C}$ , and elongation for 2 min at  $72^{\circ}\text{C}$ . The soak file was set at  $72^{\circ}\text{C}$ . DNA amplification reactions were performed in a Pharmacia LKB Gene ATAQ controller. Dilutions of leptospire in water, heated at  $100^{\circ}\text{C}$  for 10 min, were included in every amplification run as positive controls.

**Detection and characterization of PCR products.** Amplification products were separated by electrophoresis on a 2% agarose (SeaKem LE; Biozym) gel and subsequently Southern blotted (22) to a nylon membrane (Boehringer Mannheim).

To detect the PCR fragments generated by primer sets G1-G2 and B64-I-B64-II, two oligonucleotides tailed with digoxigenin (DIG)-dUTP by using the DIG oligonucleotide tailing kit (Boehringer Mannheim) were used as probes. The degenerate oligonucleotide G195-28 [5'-A(G/A)ATGATCG GCAT(T/C)ACGTT(T/C)GC(A/G)CCGTT-3'] was derived from a highly conserved sequence (nucleotides 195 to 222) between G1 and G2 (10). This oligonucleotide recognizes a broad spectrum of strains belonging to the six *Leptospira* species amplified by primer set G1-G2.

A 29-bp oligonucleotide, B88-29 (5'-CTCTGAACGATCT CATGAGTTTCCTGGAG-3'), was derived from the sequence between B64-I and B64-II (nucleotides 88 to 116). Hybridization was performed overnight at  $55^{\circ}\text{C}$  in  $5 \times (1 \times \text{SSC}$  is 0.15 M NaCl plus 0.015 M sodium citrate)-SSC-2% (wt/vol) blocking reagent (Boehringer Mannheim)-0.1% (wt/vol) *N*-lauroylsarcosine-0.02% (wt/vol) sodium dodecyl sulfate (SDS). Filters were washed twice with  $2 \times \text{SSC}$ -0.1% (wt/vol) SDS for

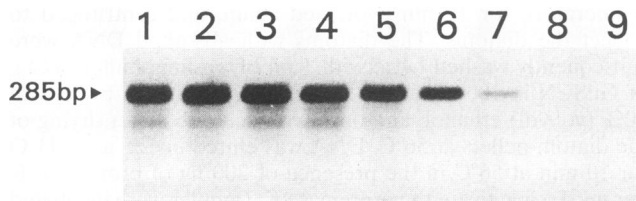


FIG. 1. Threshold of detection of leptospires in urine by PCR. Following centrifugation, DNA was extracted from the pellets of serial dilutions of urine samples seeded with *L. interrogans* Wijnberg. PCR products were generated with primer set G1-G2. Southern blot analysis was performed with DIG-tailed oligonucleotide G195-28 as the probe. Lanes: 1 to 8, serial dilutions in urine containing  $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ ,  $10^1$ , and  $10^{-1}$  leptospire(s)  $\text{ml}^{-1}$ , respectively; 9, negative control urine sample. Similar results were obtained with urine samples seeded with *L. kirschneri* Duyster by using primer set B64-I-B64-II and oligonucleotide B88-29 as the probe.

5 min at room temperature and twice with  $0.1\times$  SSC-0.1% (wt/vol) SDS for 15 min at  $55^\circ\text{C}$ . DIG-labelled probes were detected in accordance with the chemiluminescence detection protocol from Boehringer Mannheim.

## RESULTS

**Culturing of leptospires from patients' urine.** We isolated leptospires from 6 of 15 urine samples that were subjected to culturing. Two of the strains that were isolated belonged to the autumnalis serogroup, one belonged to serogroup canicola, one belonged to serogroup icterohaemorrhagiae, one belonged to serogroup celledoni, and one belonged to serogroup bataviae.

**Detection of leptospires in patients' urine by PCR.** To determine the detection limit of the procedure of DNA extraction and subsequent PCR that was used to detect leptospires in urine samples from patients, 10-fold sequential dilutions of *L. interrogans* Wijnberg and *L. kirschneri* Duyster in urine samples containing  $10^6$  to  $10^{-1}$  leptospires  $\text{ml}^{-1}$  were subjected to this procedure. After amplification with both sets of primers and subsequent Southern hybridization with oligonucleotides G195-28 and B88-29, for each strain the detection threshold was approximately 1 leptospire  $\text{ml}^{-1}$  (Fig. 1).

We found 26 of the 29 urine samples from patients with leptospirosis to be positive by PCR (Table 1). In all 26 PCR-positive urine samples, generation of a 285-bp fragment which hybridized to oligonucleotide G195-28 was observed (Fig. 2). Apparently, none of the urine samples contained *L. kirschneri* leptospires.

In all seven urine samples obtained before day 8 of illness, we were able to detect leptospiral DNA. After day 8 of illness, 19 (86%) of 22 urine samples were positive by PCR (Table 1).

TABLE 1. PCR analysis of urine samples from patients at different stages of current leptospirosis and thereafter<sup>a</sup>

PCR result	No. of samples		Total no. of urine samples
	<Day 8	$\geq$ Day 8	
Positive	7	19 <sup>b</sup>	26
Negative	0	3	3
Total	7	22	29

<sup>a</sup> All 21 negative control urine samples were PCR negative.

<sup>b</sup> Two urine samples, P1885 and P2053, were obtained more than 1 year after infection.

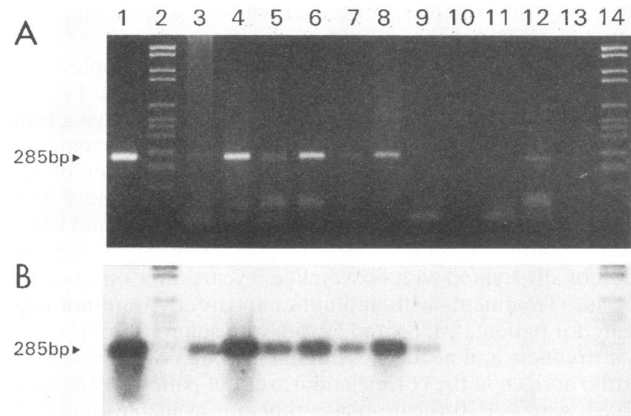


FIG. 2. Detection of leptospiral DNA in human urine by PCR. Representative PCR results obtained with DNAs extracted from urine samples from patients with leptospirosis (lanes 3 to 10) and control urine samples from healthy individuals or patients with other infections (lanes 11 to 13) are shown. Lanes: 1, *L. interrogans* Wijnberg (positive control); 2 and 14, DIG-labeled molecular size markers. Panels: A, 2% ethidium-stained agarose gel; B, corresponding Southern blot with DIG-tailed oligonucleotide G195-28.

Two of these positive urine samples had been obtained more than 1 year after infection. Including these two samples, a total of six urine samples that were positive by PCR were obtained from patients who had been treated with antibiotics at the time of illness.

In comparison with culturing, we were able to detect leptospires by PCR in twice as many freshly obtained urine samples (Table 2). In one urine sample that was culture positive, the leptospires could not be detected by PCR. The strain that was cultured from this urine sample belonged to serogroup celledoni. DNA isolated from this strain appeared to be amplified very poorly with primer set G1-G2 in our PCR (data not shown).

All aliquots of all urine and serum samples from patients with leptospirosis which were scored PCR negative were negative by PCR. The urine and serum samples used as negative controls were all negative by PCR. Except for two urine samples, all aliquots of all of the PCR-positive urine samples tested were positive. Re-extraction with GuSCN or dilution of the DNAs extracted from these two samples to eliminate possible inhibitory components of *Taq* polymerase was not effective.

**Comparison of PCR analyses of urine and serum samples for early diagnosis.** Previously, we found a detection limit of 1 to 10 leptospires  $\text{ml}^{-1}$  for PCR analysis of serum (9, 10). To compare the results of PCR analyses of urine and serum, we tested urine samples and corresponding serum samples collected before day 8 of illness from seven patients. As mentioned in the previous paragraph, all seven urine samples were

TABLE 2. Comparison of culture and PCR results obtained with freshly voided urine samples from patients with proven leptospirosis

Culture result	No. of PCR results		
	Positive	Negative	Total
Positive	5	1	6
Negative	7	2	9
Total	12	3	15

positive by PCR whereas only two of seven serum samples were positive by PCR (data not shown).

### DISCUSSION

We have explored the use of PCR analysis of urine as a diagnostic tool for leptospirosis by application of a procedure involving DNA extraction and subsequent PCR analysis of human urine samples taken at different stages of the disease.

By PCR, we were able to detect leptospires in twice as many urine samples as by culturing. Thus, PCR can be a more sensitive and rapid alternative to culturing for direct detection of leptospires in urine. A positive PCR outcome is actually only evidence of the presence of leptospiral DNA, which may originate from both viable and dead bacteria in a sample. Since leptospires do not survive long in urine, the better results obtained by PCR than by culturing may thus be attributed to the fact that PCR detected dead bacteria as well.

On the basis of results obtained by culturing, it is generally assumed that leptospiuria starts in the second week of illness (11). However, we detected leptospires in each of the seven urine samples that were taken during the first 7 days of illness. This indicates that patients shed leptospires into the urine at an early stage of illness. In four cases, PCR analysis of urine was positive before seroconversion. In the other three cases, we found low antibody titers that were not indicative of a diagnosis of leptospirosis. Thus, PCR analysis of urine can be valuable for early diagnosis of leptospirosis and, in contrast to serology, be informative in the first week of the disease.

Comparison of the PCR results obtained with urine and serum samples collected before day 8 of illness confirmed our hypothesis that use of PCR analysis of urine can be more successful than use of PCR analysis of serum.

Besides the shedding of leptospires at an early stage of the disease, we found evidence of long-term shedding, as urine samples collected from two patients more than 1 year after the acute phase of the disease were positive by PCR, suggesting persistent infection. Although the possibility of reinfection cannot be excluded, it is not likely, since no apparent rise in antibody titers was observed. Both these two patients and four other patients who were scored positive by PCR had been treated with antibiotics at the time of illness. In two cases, the positive PCR scores were confirmed by positive culture results.

The presence of leptospires in a patient's urine more than 1 year after illness indicates that leptospires can persist in the kidneys much longer than was assumed. The fact that this was observed even after treatment with antibiotics such as penicillin, amoxicillin, and vibramycin poses the question of whether the usual treatment of leptospirosis with these antibiotics is able to remove all bacteria from the human kidneys. Persistence of leptospires in the kidneys after treatment with antibiotics has been described for cattle (6, 14) and hamsters (1) and may also occur in humans (31). Persistence of leptospires is probably not caused by lack of susceptibility to antibiotics (1, 17, 20, 24), which suggests that some or most antibiotics do not reach concentrations in the kidneys which effectively eradicate leptospires. Further investigation of persistent leptospiral infections in patients, even after treatment, and possible pathogenic effects is needed. The use of a sensitive technique such as PCR may provide information about the period during which patients shed leptospires in their urine and the efficacy of treatment with certain antibiotics.

All 26 PCR-positive urine samples contained leptospiral DNA that could be amplified with primer set G1-G2. This indicates that none of these patients were infected with strains belonging to *L. kirschneri*, which was in agreement with the

results obtained by MAT, which detected no antibodies that could indicate infection with serovars of *L. kirschneri*.

Gravekamp et al. (9, 10) found no cross-reaction of the primer sets we used with several other spirochetes, various unrelated microorganisms, and human DNA. Consistently, none of the urine samples we used as controls, from patients with infections other than leptospirosis or from healthy individuals, were positive by PCR. In one case, however, an amplified DNA fragment of approximately the correct size was observed after gel electrophoresis (Fig. 2, lane 12). On a Southern blot, this amplification product did not hybridize with either of the two probes we used. Apparently, this fragment was generated as a result of mispriming. This illustrates the importance of confirmation of the specificity of the amplification product by hybridization when PCR is used as a diagnostic test.

In preliminary investigations, we established the importance of concentrating the leptospires in clinical samples to amounts that can be detected by PCR. Collecting the leptospires by centrifugation of the urine, however, can result in a concomitant concentration of factors that inhibit PCR. Earlier, Mérien et al. (18) were able to detect leptospires by PCR analysis of a small volume (100  $\mu$ l) of urine from only one patient. The method of DNA isolation they used, i.e., boiling of samples containing leptospires, gave irreproducible results in our study and inhibition of PCR when used for large amounts of urine and serum.

Although the procedure of DNA isolation with GuSCN-diatoms was developed for small quantities of urine and serum (3), we also found highly reproducible results when we applied it to large volumes of urine. For only 2 of 26 positive urine samples were we unable to detect leptospires in all of the aliquots of the same urine sample. The poor reproducibility is, in our opinion, due more to low numbers of leptospires erratically distributed, and in some aliquots just below the level of detection, than to the presence of inhibitors, since attempts to remove potential inhibitors were not effective. Furthermore, if interference with PCR was caused by the presence of inhibitors, it would likely have occurred in all aliquots of the same sample, thus rendering the entire sample PCR negative.

To monitor the effectiveness of DNA extraction and the presence of any inhibitory factors in the PCR, we are currently constructing a modified template, i.e., a recombinant plasmid containing a modified target sequence that will be coamplified. The modified template will be added to the urine samples to serve as an internal positive control.

In summary, with this PCR we describe a technique which is twice as successful as the culturing presently used to detect leptospires in urine. Furthermore, it is shown to be a promising adjunct to the early diagnosis of leptospirosis and can be useful in the study of persistence of leptospires in patients. PCR has proven to be specific for leptospirosis, more sensitive and more rapid than culturing, and in contrast to serology, informative in the first week of illness.

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