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Polymerase Chain Reaction for Detection of *Leptospira* spp. in Clinical Samples

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A sensitive assay for *Leptospira* spp., the causative agent of leptospirosis, was developed on the basis of the polymerase chain reaction (PCR). A 331-bp sequence from the *Leptospira interrogans* serovar canicola *rrs* (16S) gene was amplified, and the PCR products were analyzed by DNA-DNA hybridization by using a 289-bp fragment internal to the amplified DNA. Specific PCR products also were obtained with DNA from the closely related nonpathogenic *Leptospira biflexa* but not with DNA from other spirochetes, such as *Borrelia burgdorferi*, *Borrelia hermsii*, *Treponema denticola*, *Treponema pallidum*, *Spirochaeta aurantia*, or more distant organisms such as *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Proteus mirabilis*. The assay was able to detect as few as 10 bacteria. *Leptospira* DNA was detected in urine from experimentally infected mice. In addition, the test was found to be suitable for diagnosing leptospirosis in humans. Cerebrospinal fluid and urine from patients with leptospirosis were positive, whereas samples from control uninfected patients were negative.

Genomic DNA-DNA hybridization helped to define nine species (26) of the genus *Leptospira*, which is composed of more than 200 serovars. The nonpathogenic *Leptospira* spp. consist of three species, *L. biflexa*, *L. meyeri*, and *L. wolbachi*. The pathogenic *Leptospira* spp. consist of six species, *L. interrogans*, *L. santarosai*, *L. borgpetersenii*, *L. noguchi*, *L. weilii*, and *L. inadai* (26). Leptospirosis is a worldwide disease which affects wild and domestic animals and humans (2). In addition, some infected animal species develop persistent renal infections and shed leptospires in their urine (21). Because of the variety of clinical symptoms, leptospirosis at its onset is often misdiagnosed as aseptic meningitis, influenza, hepatic disease, or fever of unknown origin (7). Therefore, diagnosis is based on laboratory tests rather than on clinical symptoms alone. The method currently used is based on the serological response of the host to the infecting organism (enzyme-linked immunosorbent assay [ELISA] or microscopic agglutination test). The presence of leptospires can be assessed by culture; however, the process is very laborious and can take up to 2 months (7). Therefore, isolation and culture are primarily used for retrospective diagnosis. To culture the organism from tissues or body fluids, knowledge of the stage of the infection is critical. In the acute phase, which lasts for about 10 days, the leptospires can often be cultured from blood or cerebrospinal fluid (CSF). Usually, when a specific antibody response is detected (at approximately 10 days), leptospires disappear from the blood. During the second phase, which may last up to several months, bacteriuria is often intermittent.

The polymerase chain reaction (PCR) is an in vitro method for selectively amplifying specific target DNA sequences by more than 10^6 fold (20). It has been used to diagnose infectious diseases caused by fastidious or slowly growing bacteria such as *Mycobacterium leprae* (25), *Mycobacterium tuberculosis* (5), *Treponema pallidum* (10), and *Borrelia burgdorferi* (19). Two groups detected *L. interrogans* serovar hardjo in cattle urine by using the PCR (9, 22).

rRNA genes are very conserved throughout the bacterial kingdom (23). Partial rRNA sequences have been analyzed in terms of oligonucleotide "signatures" (24), which allow the definition of the major subdivisions of the eubacteria and among them the spirochete "phylum" (16, 17). These data allowed the unequivocal selection of a set of oligonucleotides within the primary structure of the *Leptospira rrs* (16S) gene (8). We report here the development of a sensitive assay for *Leptospira* DNA that is based upon amplification of the *Leptospira rrs* (16S) gene. The data suggest that the PCR assay can be used on biological samples such as CSF, urine, or blood as a diagnostic tool for cases of suspected leptospirosis.

MATERIALS AND METHODS

Organisms and growth conditions. Spirochete numbers were determined by using a Petroff-Hausser chamber. *B. burgdorferi* B31 and *Borrelia hermsii* were grown at 30°C in BSK II medium (3), while *Treponema denticola* ATCC 33520 was grown at 37°C in prereduced *T. denticola* broth (12) and *Spirochaeta aurantia* M1 was grown at 30°C in TGY medium (30 g of trypticase per liter, 20 g of yeast extract per liter, 5 g of glucose per liter [pH 7.4]). Leptospires (Pasteur Institute Collection, National Reference Center, Paris, France) were cultivated in EMJH medium (6, 13). Stock laboratory cultures were maintained by regular subculture into fresh medium, and primary isolation from clinical specimens was as described previously (7). *L. interrogans* serovar icterohaemorrhagiae strain 140 is a virulent strain maintained by serial passage in guinea pigs. All other bacteria (obtained from Pasteur Institute Hospital, Paris, France) were grown in LB broth (15).

Swiss mice were obtained from R. Janvier, Centre d'Élevage, Le Genest, Saint Berthevin, France. Two mice were infected at each inoculation. Urine samples from both mice (mean value, 25 μl each) were pooled.

Enzymatic amplification. Four oligonucleotide primers were synthesized on an Applied Biosystems DNA synthesizer (model 391) with the following sequences: A, 5'-

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GGCGGCGCGTCTTAAACATG-3'; B, 5'-TTCCCCCCAT TGAGCAAGATT-3'; C, 5'-CAAGTCAAGCGGAGTAGCA A-3'; and D, 5'-CTTAACCTGCTGCCTCCCGTA-3'.

Oligonucleotides A and B correspond to nucleotides 38 to 57 and 348 to 368 in the primary structure of the *L. interrogans rrs* (16S) gene, respectively. Oligonucleotides C and D correspond to nucleotides 58 to 77 and 328 to 347, respectively.

Amplification of DNA was performed in a total volume of 50 μl. The reaction mixture consisted of 50 mM KCl, 10 mM Tris hydrochloride (pH 8.4), 2 mM $MgCl_2$, 1 mg of gelatin per ml, 1 μM each oligonucleotide primer, and 200 μM each dATP, dTTP, dCTP, and dGTP. One unit of *Taq* DNA polymerase (Beckman Instruments, Inc., Fullerton, Calif.) was used. A 1/10-volume sample was then added, and the reaction mixture was overlaid with 50 μl of mineral oil (Sigma).

PCR was performed in a DNA thermal cycler (Gene ATAQ Controller; Pharmacia LKB). The first cycle consisted of denaturation at 94°C for 3 min, annealing at 63°C for 1.5 min, and extension at 72°C for 2 min. The next 29 cycles consisted of denaturation at 94°C for 1 min, primer annealing at 63°C for 1.5 min, and extension at 72°C for 2 min (an additional 10 min was included at the end of the cycles to complete extension of the primers).

Preparation of samples for PCR analysis. (i) Pure leptospires. Cultured leptospires from the Pasteur Institute Collection were tested by PCR. Serial 10-fold dilutions of leptospires in sterile distilled water were collected by centrifugation at 13,000 × *g* for 15 min at 4°C. The pellets, washed twice with 100 μl of water, were resuspended in 10 μl of TE buffer and heated at 96°C for 10 min.

(ii) Purified *Leptospira* DNA. The *Leptospira* DNA was extracted and purified as described by Brenner et al. (4).

(iii) Reconstitution experiments with biological samples. To evaluate the method of sample preparation, artificial inoculation of biological samples was performed before PCR assay. The method for preparing the samples was as follows. CSF samples from patients with diseases other than leptospirosis were seeded with leptospires and used for PCR. Samples were centrifuged and washed as described above except that a single wash was used. Concentration and heat treatment were as described above. Two batches of CSF, seeded with serially diluted *L. interrogans*, were stored for 48 h at 4°C (first batch) or at room temperature (second batch).

Leptospires were mixed with urine from a healthy human and tested by PCR. These samples were first centrifuged at 800 × *g* for 10 min at room temperature to eliminate large particles (bladder cells and urinary crystals and cylinders, etc.) and then washed, concentrated, and heat treated as described above. The first two batches were stored for 48 h at either 4°C or room temperature. Formaldehyde (final concentration, 0.5%) was added to the third batch before the batch was stored for 48 h at room temperature.

Leptospires were mixed with heparin-anticoagulated whole blood from a healthy human and tested for PCR. The tubes containing 1 ml of blood were centrifuged at 800 × *g* for 15 min at room temperature, and 0.5 ml of plasma was removed. After centrifugation of the plasma at 13,000 × *g* for 10 min at 4°C, the pellets were washed twice in 100 μl of sterile distilled water, suspended in 10 μl of sterile filtered TE buffer, and heated at 96°C for 10 min.

(iv) Shedding state of mice. The precocity of the shedding state of mice experimentally infected by intraperitoneal infection with 10^3 to 10^6 *L. interrogans* serovar icterohaemorrhagiae strain 140 organisms was assayed by PCR. Urine was collected 4 to 10 days after infection. When mice are picked by the experimenter, they pass drops of urine which remain on the hair and can be collected with an automatic pipette.

(v) Biological samples from patients. Urine, CSF, or blood (100 μl of each) from 28 patients were tested by PCR. The samples were received by the laboratory to be tested for diverse infectious diseases including leptospirosis. Six patients were clearly seropositive as determined by microscopic agglutination test or ELISA (either a rise in the antibody levels in a series of specimens from one patient or a unique titer lower than 1/800). Twenty-two negative control samples (13 CSF, 2 blood, and 7 urine samples) were seronegative (repeated results) and culture negative, and the final biological diagnosis excluded leptospirosis. The biological samples were treated as described above for the reconstitution experiments.

Detection of PCR products. The amplification products (10 μl or one-fifth of the reaction mixture) were analyzed by electrophoresis in a 1.5% agarose gel (stained with ethidium bromide). The molecular size markers were pBR328 digested by *Bgl*I and *Hin*fI (Boehringer, Mannheim, Germany) of the following sizes: 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220, and 154 bp.

For greater sensitivity as well as specificity, analysis of the PCR products was routinely performed by hybridization with a probe specific for *Leptospira* 16S RNA. The C and D primers internal to oligonucleotides A and B used for the original amplification were used to synthesize this probe (289 bp) which was synthesized in a PCR where dTTP was replaced by digoxygenin-11-dUTP (Boehringer).

PCR products (10 μl) were added to 40 μl of TE buffer and boiled for 10 min. The samples were immediately put on ice and then applied to a nylon membrane (Hybond-N, Amersham) by using vacuum suction. The filters were dried, baked for 2 h at 80°C, and hybridized with the *L. interrogans* probe as described in the Boehringer manual. Detection of the digoxygenin-labeled probe was by use of anti-digoxygenin-alkaline phosphatase as substrate.

RESULTS

Specificity and sensitivity of PCR amplification. PCR was used to detect *Leptospira* DNA by using the *rrs* (16S) gene as a target sequence. We first analyzed by PCR the 20 serotypes that are used routinely in our diagnostic laboratory as the antigens encompassing most of the known cross-reactions of leptospires for the microscopic agglutination test (the first 20 *Leptospira* strains shown in Table 1). Each serotype produced a positive signal (a 331-bp band in agarose gel electrophoresis). An example of five different serovars is given in Fig. 1. One representative of each available pathogenic species as defined by Yasuda et al. (26) and the nonpathogenic *L. biflexa* serovar patoc also produced the same-size DNA product (Table 1). The sensitivity of the reaction was determined for *Leptospira* DNA ranging from 1 ng to 10 fg in amount. The lower limit of detection of *Leptospira* DNA in the assay was 1 pg by agarose gel electrophoresis and 10 fg by DNA hybridization (which correspond theoretically to two bacteria) (Fig. 2).

To determine the specificity of the oligonucleotide primers for *Leptospira* spp., a number of pathogenic organisms were tested by using these primers and the same hybridization conditions for PCR. *B. burgdorferi*, *B. hermsii*, *T. denticola*, *Treponema pallidum*, *S. aurantia*, *Escherichia coli*, *Shigella*

TABLE 1. List of *Leptospira* strains used for PCR

Serogroup	Serovar	Strain	Species[a]
Australis	australis	Ballico	*L. interrogans*
Autumnalis	autumnalis	Akiyami A	*L. interrogans*
Ballum	castellonis	Castellon 3	*L. borgpetersenii*
Bataviae	bataviae	Van Tienen	*L. interrogans*
Canicola	canicola	Hond Utrecht IV	*L. interrogans*
	canicola	Chiffon	*L. interrogans*
Cynopteri	cynopteri	3522C	*L. alstoni*[b]
Grippotyphosa	grippotyphosa	Moskva V	*L. alstoni*[b]
Hebdomadis	hebdomadis	Hebdomadis	*L. interrogans*
Icterohaemorrhagiae	copenhageni	Wijnberg	*L. interrogans*
	icterohaemorrhagiae	Verdun	*L. interrogans*
Javanica	javanica	Veldrat Batavia 46	*L. borgpetersenii*
Panama	panama	CZ214	*L. noguchi*
Pomona	pomona	Pomona	*L. interrogans*
Pyrogenes	pyrogenes	Salinem	*L. interrogans*
Sejroe	hardjo	Hardjoprajitno	*L. borgpetersenii*
	serjoe	M84	*L. borgpetersenii*
	wolffi	3705	*L. borgpetersenii*
Semaranga	patoc	Patoc I	*L. biflexa*
Tarassovi	tarassovi	Mitis Johnson	*L. borgpetersenii*
Sarmin	sarmin	Sarmin	*L. weilii*
Shermani	shermani	1342K	*L. santarosai*

[a] As defined by Yasuda et al. (26).
[b] Reported by Kaufmann (14).

flexneri, *Salmonella enteritidis*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Streptococcus* group D, *Staphylococcus aureus*, and *Mycobacterium tuberculosis* were all negative. No amplification products were detected with non-*Leptospira* DNA by either agarose gel electrophoresis or DNA hybridization (data not shown).

Precocity of the shedding state of mice experimentally infected with *L. interrogans*. Urine from Swiss mice that were experimentally infected with 10^3 to 10^6 cells of *L. interrogans* serovar icterohaemorrhagiae strain 140 was analyzed by both classical dark-field microscopy and PCR. Culture in EMJH medium was not attempted because of the contamination of urine samples by common microorganisms and the tiny volumes of the specimens. Samples obtained before infection or 4 to 10 days after infection were amplified, and the products of amplification were analyzed by hybridization (Fig. 3). No amplification products were detected in samples from the uninfected mice. In mice that were inoculated with 10^3 pathogenic leptospires, a positive signal in a dot blot was obtained after 8 days. In mice that were inoculated with 10^6 leptospires, a positive signal in a dot blot was obtained only 4 days after inoculation. Thus, the onset of shedding of leptospires seems to be chronologically related to the size of the inoculum.

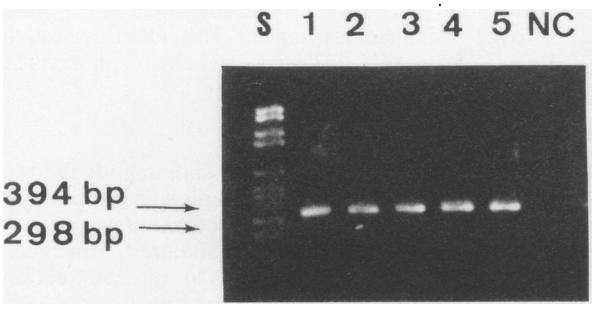

FIG. 1. Example of PCR amplification of five *L. interrogans* serovars demonstrated by agarose gel electrophoresis after ethidium bromide staining. One nanogram of template DNA was used in the PCR (30 cycles with primers A and B). Lanes: 1 to 5, *L. interrogans* serovar castellonis strain Castellon 3, serovar australis strain Ballico, serovar tarassovi strain Mitis Johnson, serovar bataviae strain Van Tienen, serovar sejroe strain M84, respectively; NC, negative control without DNA; S, pBR328 cleaved with *BglI* and *HinfI* used as a DNA size marker (in base pairs).

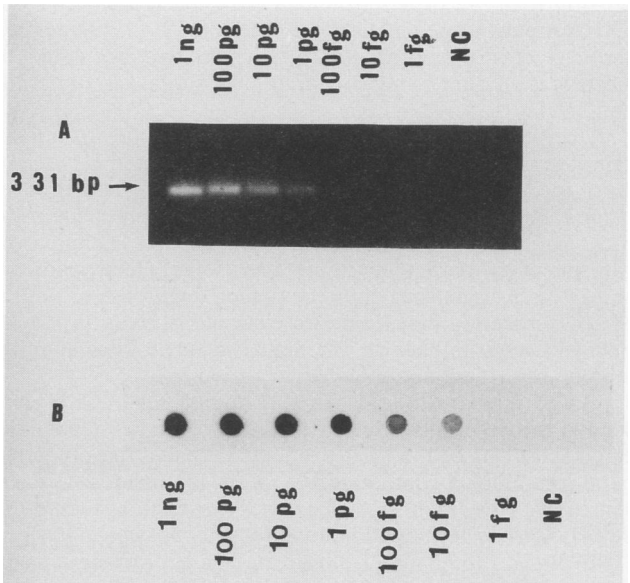

FIG. 2. Comparison of sensitivity of the PCR assay (30 cycles with primers A and B) for purified *L. interrogans* serovar icterohaemorrhagiae strain 140 DNA after agarose gel electrophoresis stained with ethidium bromide (A) and dot blot hybridization with a digoxygenin-labeled probe (B). The amounts of DNA used in each PCR are shown above and below the lanes. NC, negative control without DNA.

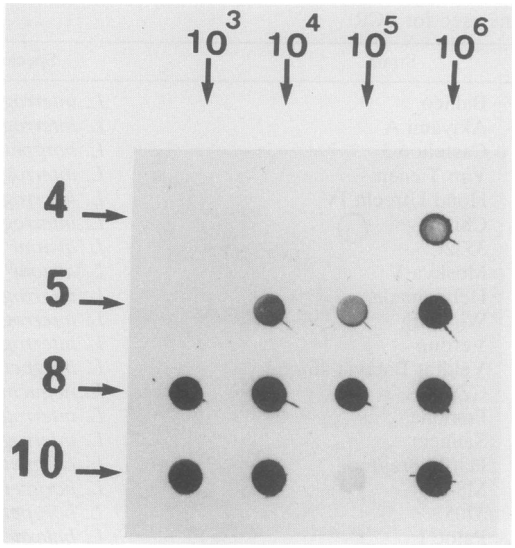

FIG. 3. Dot blot hybridization with a digoxygenin-labeled probe of urine specimens of Swiss mice intraperitoneally infected with a pathogenic *L. interrogans* serovar icterohaemorrhagiae strain 140. Specimens were prepared and subjected to amplification as described in the text by using primers A and B for 30 cycles. Days after infection and numbers of inoculated pathogenic leptospires are indicated to the left and at the top of the figure, respectively.

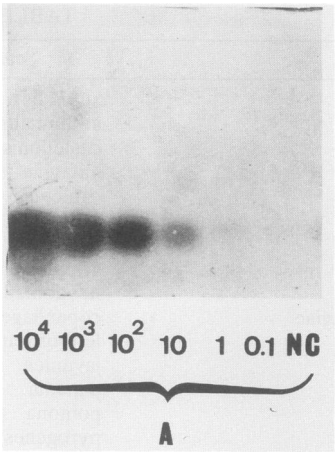

FIG. 4. Southern blot hybridization with a digoxygenin-labeled probe of PCR products (30 cycles with primers A and B) of urine specimens seeded with serially diluted *L. interrogans* serovar icterohaemorrhagiae strain 140. Urine was stored for 48 h at room temperature. The number of organisms per milliliter is indicated. NC, negative control without DNA.

In comparison, detection of PCR products by direct agarose gel electrophoresis was possible from day 8 with all inocula and from day 5 with the largest inoculum. With dark-field microscopy, leptospires could be detected only from day 8 with an inoculum of 10^6 bacteria. These results indicate that PCR is more sensitive than dark-field microscopy in detecting infection.

Biological samples mixed with leptospires. To evaluate the sensitivity of the PCR for biological samples, human blood, CSF, and urine specimens were seeded with *L. interrogans* serovar icterohaemorrhagiae strain 140 and subjected to amplification after being prepared as described in Materials and Methods.

(i) Urine mixed with leptospires. Three batches of human urine were seeded with serially diluted cultivated *L. interrogans* to determine the effects of temperature and time of incubation on the preservation of leptospires in urine samples. For the three urine batches, after PCR, 100 microorganisms could be detected with agarose gel electrophoresis and 10 microorganisms could be detected with dot or Southern hybridization detection. An example is given in Fig. 4. Formaldehyde intensified the signal in urine kept at room temperature (data not shown).

(ii) CSF mixed with leptospires. Two batches of CSF were seeded with serially diluted *L. interrogans*. Agarose gel electrophoresis of the PCR products revealed the predicted band for samples containing 10^2 to 10^5 leptospires. Greater sensitivity was obtained when dot hybridization was used; detection of as few as 10 leptospires was possible.

(iii) Blood mixed with leptospires. The heparin-anticoagulated whole blood seeded with *L. interrogans* was subjected to PCR immediately after treatment (see Materials and Methods). One hundred leptospires could be detected by a visible band on the agarose gel compared with 10 leptospires by hybridization.

Amplification of leptospiral DNA in biological samples from patients with leptospirosis. A total of 28 samples (urine, CSF, or blood) were tested for the presence of *Leptospira* DNA. The samples originated from six patients with suspected leptospirosis and 22 negative controls. The 22 negative control samples were PCR negative.

The clinical characteristics and serological and PCR results of the six patients (A to F) with suspected leptospirosis are shown in Table 2. Four of these six were PCR positive by hybridization. The experiment was repeated, with identical results.

Among the PCR-negative cases, one patient (patient E) had a low but significative antibody titer by the microscopic agglutination assay (1/800), which could reflect a leptospirosis initiated more than 1 month earlier. This hypothesis could explain a negative PCR result as a consequence of an absence of leptospires in the CSF sample. Patient F died, no doubt from icterohaemorrhagic leptospirosis. The negative PCR result in this case could be explained by a blood specimen taken late in infection (day 14 after the onset of the infection) and heavy antibiotic treatment.

Isolation of *Leptospira* spp. from the clinical specimens of the six seropositive patients was attempted. For patient C only, leptospires were isolated and identified as *L. interrogans* serovar icterohaemorrhagiae. This identification was made by use of pulsed-field agarose gel electrophoresis (11).

DISCUSSION

Detection of pathogenic *Leptospira* spp. in body fluids and tissues is important for diagnosing leptospirosis because it provides unequivocal evidence of active infection. Widespread application of this strategy is hindered, however, by the limitation of conventional methods for direct detection of pathogenic leptospires in clinical specimens. As a consequence, clinical decision making is often based upon serological tests despite the fact that antibodies begin to appear 8 to 10 days after the onset of the illness. We used PCR to develop a highly sensitive and specific assay for *Leptospira* spp. to help in the detection and treatment of infection at an early stage.

DNA-DNA hybridization with the *rrs* (16S)-specific probe was used routinely as a means of enhancing specificity as

TABLE 2. Comparison of methods for the detection of leptospires in human patients with clinical and epidemiological history

Patient	Age (yr)	Sex	Clinical and epidemiological history	Serologic results	Culture results	PCR results (sample)
A	17	M	Fever (40°C), coma; exposure to domestic animal (dog), fishing in lake	+	−	+ (CSF)
B	24	M	Fever (38.5°C), meningitis; bathing in river, travel to Thailand	+	−	+ (urine)
C	18	M	Fever (39–40°C), algia, stiff neck, icterus; bathing in river	+	+	+ (CSF)
D	68	F	No fever, meningitis; gardening	+	−	+ (CSF)
E	57	M	Epistaxis, renal insufficiency, respiratory insufficiency, hepatic cirrhosis, chronic alcoholism	+	−	− (CSF)
F	40	M	Fever (39°C), respiratory insufficiency, icterus, thrombopenia, renal insufficiency, intensive purpura; diving	+	−	− (blood)

well as sensitivity. Recognition of pathogenic and nonpathogenic *Leptospira* spp. irrespective of serovar was obtained. The inability to distinguish between the saprophytic *L. biflexa* and pathogenic *Leptospira* spp. by amplification of the *L. interrogans rrs* (16S) gene, as performed in this study, is not of practical consequence since *L. biflexa* was usually not found in human samples (7; also our unpublished results). It is noteworthy that the PCR assay developed by us can readily distinguish between *L. interrogans* and *B. burgdorferi*; cross-reactivity between the two spirochetes has been reported in serodiagnostic tests for leptospirosis and Lyme disease (18).

The two other reports of detection of *Leptospira* spp. concerned the specific identification of serovar hardjo in bovine urine (9, 22), whereas our study reports the detection of *Leptospira* spp. in clinical samples irrespective of serovar.

The isolation of *Leptospira* spp. was successful in one of six clinical specimens, while four of the six were positive by PCR. The data presented here indicate that the PCR provides a technique that could surpass culturing in sensitivity of detection. Experiments with purified DNA indicated that a very small number of leptospires can be detected by the PCR. In blood, CSF, and urine seeded with leptospires, the sensitivity was very high since as few as 10 bacteria gave a clear signal detectable by hybridization. This high sensitivity was confirmed by the results of the comparative study of three different techniques. Our results showed that the PCR is faster than culturing for isolating *L. interrogans* (1 day versus 1 to 8 weeks). The rapid detection of leptospires at an early stage of the disease may favorably influence the course of the disease.

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