JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1989, p. 2258–2262
0095-1137/89/102258-05$02.00/0

Vol. 27, No. 10

# Detection of Leptospires in Urine by Polymerase Chain Reaction

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Received 23 March 1989/Accepted 12 June 1989

**Primers for polymerase chain reaction (PCR) were synthesized from clones derived from a *Leptospira hardjo* (type hardjobovis) library. One pair of synthetic oligonucleotide primers was selected for further analysis. Under experimental conditions an amplification was obtained with DNA of *Leptospira interrogans* of some serovars belonging to serogroup sejroe. However, very little or no amplification was observed with DNA from other serovars of this group. No amplification was observed with DNA from other serogroups, other bacteria, or eucaryotic organisms. Cattle urine, seeded with hardjobovis, was processed in several ways and subsequently subjected to PCR. Boiling of the samples or treatment with detergents appeared to be most effective. Urine samples containing fewer than 10 leptospires gave a positive result in the PCR assay. Twenty urine samples obtained from a slaughterhouse or farm cows were investigated using the PCR assay, culture isolation, dot and quick blot hybridization, and serological tests. This comparative study suggests that amplification by PCR may be a valuable method for the detection of leptospires in cattle urine.**

The detection and characterization of leptospires are currently done with culture isolation and serological techniques. Culture is slow, laborious, and susceptible to contamination. In human infections serological tests become positive only several days after the onset of illness. In chronic animal infections serology does not provide reliable information on the carrier or shedding state.

DNA-based techniques have been introduced into the field of leptospirosis. They have been demonstrated to provide a useful addition and alternative in the characterization of leptospiral strains (6, 8, 18, 19). Techniques such as restriction endonuclease analysis and Southern blotting may be suitable for identification, but they are time-consuming and laborious (3, 17, 19). In situ and dot blot hybridizations may be useful for routine diagnosis, but are probably no more sensitive than immunological or immunohistochemical methods (15, 16, 21).

For the diagnosis of leptospirosis, an assay is needed that is able to detect small numbers of leptospires in blood or urine and that can be performed within a short period of time. In this way an infection can be detected and treated at an early stage. For epidemiological studies such an assay should be sensitive and allow many samples to be processed simultaneously. The polymerase chain reaction (PCR) assay may fulfill these requirements. In studies of genetic disorders and viral infections, PCR has been demonstrated to be both sensitive and rapid (1, 5, 9–11, 13, 20). The specificity of the assay can be adjusted by the choice of primers.

In the present study PCR was applied to urine samples from cattle infected with *Leptospira hardjobovis*. This may also serve as a model for other leptospiral infections. The data suggest that the PCR assay can be used as a tool for diagnosis as well as for large-scale epidemiological studies.

## MATERIALS AND METHODS

**Leptospiral strains and urine samples.** Reference strains, obtained from the World Health Organization/Food and Agricultural Organization culture collection in our institute, were cultured as described by Johnson and Harris (4). In the case of hardjobovis cultures, the medium was enriched with 1% fetal calf serum plus 1% rabbit serum. Urine samples were obtained sterilely from slaughterhouse cows and from infected farm cows (kindly provided by A. B. Bokhout). Hardjobovis-negative control urine (from a cow kept under controlled conditions) was also provided by Bokhout.

**Molecular biological techniques.** Preparation of chromosomal DNA, endonuclease digestion, and cloning of leptospiral DNA fragments have been described previously (19). All procedures were performed basically according to Maniatis et al. (7). Southern blotting was, with minor modifications, carried out as described by Southern (14). DNA fragments were sequenced directly from plasmids by the method developed by Sanger and co-workers (12), using a Boehringer Mannheim sequencing kit.

**Sample preparation.** Leptospires from cultures or cattle urine were collected by centrifugation at 13,000 × *g* for 15 min at 4°C. For the samples that were seeded with leptospires, the leptospires were counted microscopically. Pellets were suspended in distilled water or urine, and serial 10-fold dilutions were made. Samples were centrifuged again for 15 min at 13,000 × *g*, and pellets were resuspended. An additional washing step appeared to have no effect on the efficiency of amplification. DNA was released from leptospires by one of the following methods: (i) sonication of suspended leptospires in 0.5 ml of distilled water by a 23-s dicontinuous 60-W pulse (Branson Sonifier B15); (ii) sonication as above, followed by purification of DNA by Geneclean (Bio 101 Inc.) according to the instructions of the manufacturer; (iii) incubation of bacteria in 30 μl of buffer containing 50 mM KCl, 10 mM Tris hydrochloride (pH 8.3), 2.5 mM $MgCl_2$, 60 μg of proteinase K per ml, 0.45% Nonidet P-40, and 0.45% (wt/vol) Tween 20 at 55°C for 1 h (M. T. E. Cornelissen, J. G. Van den Tweel, A. P. H. B. Struijk, M. F. Jebbink, J. A. Van der Noorda, and J. Ter Schegget, in press); (iv) heating of leptospires suspended in water for 10

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1                                                                    60
GATCNNTTCT CGGGNAGTTT CCTGCAAATG TTANAGGATG TATTCCGTAA GTTCGTTTCC
CTAGNNAAGA GCCCATCAAA GGACGTTTAC AATNTCCTAC ATAAGGCATT CAAGCAAAGG

61
********** **********                                           120
CGGTTCGTAA AACGAGACAA GTGCTCCCGT TACCGAAACC CAGAAGACCC TGTTTAAACT
GCCAAGCATT TTGCTCTGTT CACGAGGGCA ATGGCTTTGG GTCTTCTGGG ACAAATTTGA


121                                                                 180
TTGATATGGG GCGTTGTCTC GGCCCTTGCC AAGGAAATAT TCCCGTAGAA GATTATAAAA
AACTATACCC CGCAACAGAG CCGGGAACGG TTCCTTTATA AGGGCATCTT CTAATATTTT


181                                                                 240
TTGTAATCGA TCAAGTGATT CAATTCTTGG AAGGTAAAAA AGAATCTCTT GTGGGGGATC
AACATTAGCT AGTTCACTAA GTTAAGAACC TTCCATTTTT TCTTAGAGAA CACCCCCTAG


241                                                                 300
TCAGTATCAA AATGAGTGCG TCGTCGAATC GGATGGATTT TGAAAAGCCG CTCGTTATCG
AGTCATAGTT TTACTCACXC AXCAGCTTAG CCTACCTAAA ACTTTTCGGC GAGCAATAGC
                                  ********** **********

301                                                                 360
GATATCGTCA GCGAATTCGA GCTCGGTACC CGGGATCTCT AGAGTCGACT GCGCTCACTG
CTATACGAGT CGCTTAAGCT CGAGCCATGG GCCCTAGAGA TCTCAGCTGA CGCGAGTGAC


361                                                          408
CTATACGTTA GCGTCGGGAT GTACGAACTC CACTCGCGAGC TAGTAGC
GATATGCAAT CGCAGCCCTA CATGCTTGAGG TGAGCGCTCG ATCATCG
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FIG. 1. Sequence of clone pLBEc23s derived from a genomic library of *Leptospira interrogans* serovar hardjo, type hardjobovis, strain HB013. The sequences used for the oligonucleotide primers are marked by asterisks.

min at 100°C. All samples were either stored at −20°C or immediately subjected to PCR.

**DNA sequence amplification (PCR).** DNA preparations or urine samples were heated to 96°C for 10 min and quenched on ice to destroy any residual enzymatic activity and as a first denaturing step. Amplification of DNA was performed in a total volume of 100 μl. The reaction mixture contained 10 μl of a 10× reaction buffer (50 mM KCl, 20 mM $MgCl_2$, 100 mM Tris hydrochloride [pH 8.3], and gelatin at a final concentration of 100 μg/ml). Primers were used at a final concentration of about 100 pM, and the four deoxynucleotide triphosphates (Pharmacia) were used at a concentration of 100 μM. To each sample, 1 U of *Taq* polymerase was added (purchased from Perkin-Elmer Cetus). The samples were covered with 100 μl of mineral oil and placed in an automatic PCR processor (Biomed Instruments, Inc.). One amplification cycle consisted of annealing of primers for 2 min at 55°C, elongation for 3 min at 72°C, and denaturation for 2 min at 94°C. The number of cycles was varied from 20 to 40, but unless indicated otherwise, 32 cycles were used. The last elongation step was extended to 7 min. After amplification a 20-μl portion of each sample was subjected to electrophoresis on a 2% agarose gel. Gels were photographed and blotted under alkaline conditions (0.4 N NaOH) onto nylon filters (Zetaprobe; Bio-Rad). Filters were hybridized either to random primed probes that consisted of the fragment between the primers or to the whole plasmid containing this fragment. Hybridization conditions have been described previously (19).

**Oligonucleotide primers.** Oligonucleotide sequences were obtained from clone pLBEc23s from a hardjobovis library (Fig. 1). Primers were synthesized in an Applied Biosystems DNA synthesizer. Optimal amplification conditions for the desired specificity of the oligonucleotides were determined in PCR assays by using purified DNA of serovars belonging to serogroup sejroe, such as hardjo, types hardjobovis and hardjoprajitno, sejroe, wolffi, and balcanica; a number of serovars belonging to other serogroups, such as icterohaemorrhagiae (serovars copenhageni and lai) and autumnalis (butembo); and nonpathogenic leptospiral serovars, such as patoc and andaman. The primers were further tested for specificity on bacteria related to leptospires, such as *Borrelia burgdorferi* and *Treponema reiteri*, and on evolutionarily more distant organisms, such as several mycobacteria, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Salmonella* sp. group B, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, and *Escherichia coli*. *Leishmania*, mouse, rat, and human DNA was also tested. Pure DNA preparations were either obtained from other groups in this laboratory or extracted as previously described (19). The specificity of reactions was determined for starting DNA quantities of 0.1 to 100 ng.

## RESULTS

Clone pLBEc23s, from which the sequences for the oligonucleotides were derived, was selected on the basis of dot blot and Southern hybridization characteristics. Only one copy of the sequence represented by this clone appears to be present in the hardjobovis genome. Clone pLBEc23s showed a strong hybridization with its homologous serovar hardjo, type hardjobovis, and with serovars balcanica, sejroe, polonica, dikkeni, nyanza, and saxkoebing. Weaker or undetectable hybridization was found with other serovars belonging to serogroup sejroe (Fig. 2) or with serovars belonging to other serogroups. Sequencing of pLBEc23s did not reveal any structural sequences, such as inverted repeats, that might interfere with *Taq* polymerase elongation. The selected DNA sequence contained 200 base pairs between the primers; the G+C content was about 40%. A computer search was done for homologies with a DNA sequence data base, but no significant homologies were found

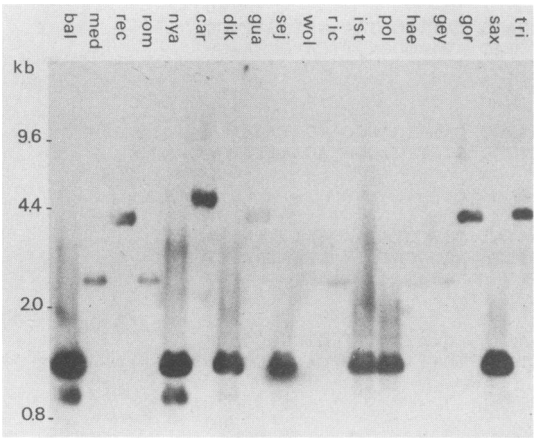


FIG. 2. Autoradiogram of a Southern blot of serovars belonging to serogroup sejroe. Four micrograms of leptospiral DNA was digested with *Eco*RI and fractionated on a 0.7% agarose gel. The Southern blot was hybridized with probe pLBEc23s. Abbreviations for serovars: tri, trinidad; sax, saxkoebing; gor, gorgas; gey, geyaweerda; hae, haemolytica; pol, polonica; ist, istrica; ric, ricardi; wol, wolffi; sej, sejroe; gua, guaricurus; dik, dikkeni; car, caribe; nya, nyanza; rom, romanica; rec, recreo; med, medanensis; bal, balcanica.

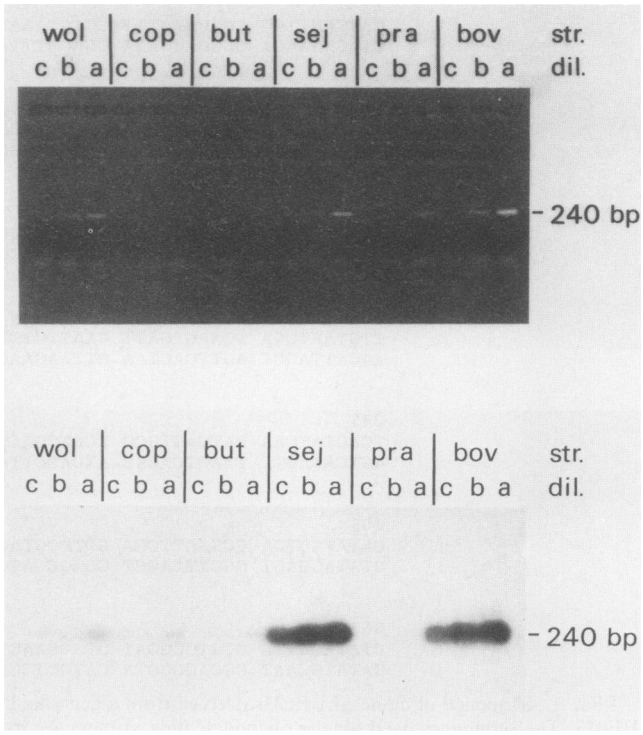


FIG. 3. Photograph of an agarose gel and autoradiogram of a Southern blot of this gel. The blot was hybridized to probe pLBEc23s. Serial dilutions (10 [a], 1 [b], and 0.1 [c] ng) of DNA of several serovars were subjected to PCR. Bands on the agarose gel are weak for serovars wolffi (wol) and hardjo, type hardjoprajitno (pra). No bands are observed for serovars copenhageni (cop) and butembo (but), belonging to serogroups icterohaemorraghiae and autumnalis, respectively. Relatively strong bands on the agarose gel are observed for serovars hardjo, type hardjobovis (bov), and sejroe (sej). A strong hybridization is found only for hardjobovis and the closely related serovar sejroe. bp, Base pairs; str., strain; dil., dilution.

To determine the sensitivity of PCR and the specificity of the primers, several combinations of annealing temperature and number of amplification cycles were tested. Under an annealing temperature of 42°C, most serovars of serogroup sejroe produced a clear band on agarose gels, but hybridization with an internal probe showed a hybridization signal only with serovars that strongly reacted with probe pLBEc23s (see above). Very little or no hybridization was found for other serovars. Higher annealing temperatures decreased amplification of DNA from serovars such as wolffi, trinidad, and hardjoprajitno. A primer annealing temperature of 55°C and 32 amplification cycles appeared to provide good assay conditions. Under these conditions 0.1 ng of DNA from serovars such as hardjo (type hardjobovis) and sejroe was amplified to a level clearly visible on agarose gels (Fig. 3). Larger quantities are needed to produce an equally strong band for serovars such as hardjo (type hardjoprajitno) and wolffi (Fig. 3). On Southern blots 0.01 pg of DNA from hardjobovis or closely related serovars, such as balcanica and sejroe, could be detected, whereas 10 ng of DNA from other serovars produced a very weak or no signal (Fig. 3). When 20 amplification cycles were used, the bands on the agarose gels were very faint and differences in amplification between serovars could be determined only after Southern blotting. After more than 40 cycles additional bands were observed that were not the correct size and did not hybridize to pLBEc23s. There was no amplification for serovars belonging to other serogroups or for other bacteria or eucaryotes (Fig. 4).

Leptospires belonging to serovar sejroe or hardjo, type hardjobovis, were seeded in medium or urine, and three methods of sample preparation were investigated: (i) treatment of leptospire-containing medium or urine with the detergents Tween 20 and Nonidet P-40 gave the best results; (ii) sonication of samples, followed by Geneclean DNA purification, gave erratic results; (iii) boiling for 10 min gave satisfactory results (although amplification was somewhat less than after detergent treatment, boiling is a simpler and more economical method). With either the detergent or the boiling method 10 to 20 hardjobovis leptospires produced, after PCR (55°C and 32 cycles), a visible band on agarose gels. Fewer than 10 leptospires could be detected by Southern blotting. If the boiling method is used, inhibitors in urine may reduce the sensitivity, but this inhibition was only

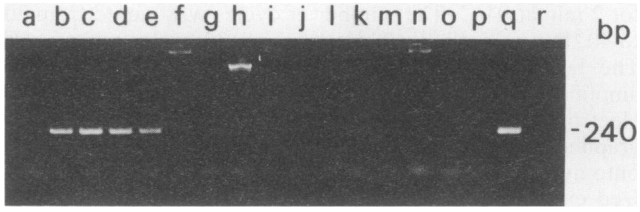


FIG. 4. Photograph of an agarose gel of a PCR performed on a sample containing no DNA (a and r), on samples containing 10 ng of DNA isolated from leptospiral serovars hardjo, type hardjobovis (two isolates) (b and c), sejroe (two isolates) (d and e), and balcanica (q), and on bacteria other than leptospires: (f) *Mycobacterium bovis*, (g) *Mycobacterium avium*, (h) *Mycobacterium peregrinum*, (i) *E. coli*, (j) *Salmonella* sp. group B, (k) *Y. enterocolitica*, (l) *S. pneumoniae*, (m) *K. pneumoniae*, (n) *P. aeruginosa*, (o) *B. burgdorferi*, and (p) *Treponema reiteri*. The high band observed for *Mycobacterium peregrinum* is more than 1 kilobase in size and does not hybridize with pLBEc23s. bp, Base pairs.

TABLE 1. Comparison of methods for the detection of *leptospires* in cattle urine

| Sample | Origin | Detection by: | | | |
|---|---|---|---|---|---|
| | | Serology | Culture | Quick blot | PCR |
| CDI-00[a] | Lab | − | − | − | − |
| CDI-06[b] | Farm | + | + | + | + |
| CDI-08[b] | Farm | + | + | + | + |
| KIT-2704 | SH[c] | − | − | − | + |
| KIT-2705 | SH | − | − | − | + |
| KIT-2706 | SH | − | − | − | + |
| KIT-2707 | SH | − | − | − | − |
| KIT-2709 | SH | − | − | − | − |
| KIT-2710 | SH | − | − | − | − |
| KIT-2712 | SH | − | − | − | − |
| KIT-2713 | SH | − | − | − | − |
| KIT-2752 | SH | − | − | − | − |
| KIT-2753 | SH | − | − | − | − |
| KIT-2943 | SH | + | + | − | + |
| KIT-2944 | SH | + | + | − | + |
| KIT-2946 | SH | + | + | − | + |
| KIT-2952 | SH | + | + | − | + |
| KIT-2953 | SH | + | + | − | + |
| KIT-2954 | SH | + | + | − | + |
| KIT-2980 | SH | + | − | − | + |
| KIT-2981 | SH | + | − | − | + |

[a] Cow that had never been outside the laboratory; the chance of inadvertent infection was considered to be negligible (negative control).
[b] CDI-06 and CDI-08 were positive controls.
[c] SH, Slaughterhouse.

observed in the PCR mixture containing a high concentration (10%) of urine. After 10-fold dilution of the urine, the inhibition was no longer observed.

Urine samples from serologically positive and negative cows were screened by quick blot, culture, and PCR. PCR revealed the presence of leptospires in the urine of all cows positive by the culture or serological tests. Of the 10 serologically negative cows, 3 were positive by PCR assay (Table 1). Only a few of the serologically positive cows were found to be positive by dot blot or quick blot assay (Table 1). There was no evidence for disturbance of the test by contaminating microorganisms that were possibly present. Negative controls, blanks, and confirmed negative urine samples were negative in all tests.

## DISCUSSION

The detection of leptospires at an early stage of infection increases the chance of successful treatment and will reduce pathology in human infections. In cattle it will provide an opportunity to reduce or prevent the spread of infection by culling. At present there are no rapid and practical means for the early detection of leptospiral infections. The data presented show that PCR provides a technique that surpasses the sensitivity of assays explored so far. Experiments with purified DNA indicated that a very small number of leptospires can be detected. Media and urine seeded with leptospires showed that the sensitivity is very high since 10 to 20 bacteria gave a clear signal on agarose gels. The high sensitivity was confirmed by the results of the comparative study of four different detection techniques. Our results with a number of cattle urine samples showed that PCR is at least as sensitive as culturing or serological methods and far more sensitive than DNA-based techniques such as dot and quick blot hybridizations. Serology does not necessarily reflect the carrier or shedding state of cattle (2). Positive results by PCR in otherwise negative urine samples of seronegative cows may indicate a high sensitivity rather than being false-positive. However, these results should be considered with caution until large-scale studies have been performed.

In addition to sensitivity and specificity, labor and time needed for the assay and the complexity of the procedure will determine the usefulness of PCR as a detection assay for leptospires. PCR will only work as a diagnostic tool when results can be obtained within 1 day. It will only work as an epidemiological tool if a large number of samples can be processed simultaneously. Thus, because of the savings in time and simplicity, simply boiling the leptospire-containing samples seems to be the most attractive method. Inhibitory compounds in urine that are trapped by this procedure may interfere with amplification, but the sensitivity of PCR is so high that inhibition at a high urine concentration can be nullified by simple dilution of the sample (1). The urine samples used in this study had been stored for months. Storage and freeze-thawing may affect the quality of the DNA, but this apparently has no effect on amplification, as was previously shown for cytomegalovirus (1). This is a considerable advantage over culture, which can only reveal the presence of viable leptospires.

The specificity of the PCR assay is determined by the annealing efficiency of the primers, which depends on their homology with the target DNA and the temperature of the annealing step. An increase in the annealing temperature allows fewer mismatches between primers and target DNA and will increase the specificity of annealing. As the number of amplification cycles increases, the chances of synthesis of unwanted products increase. Therefore, we chose a relatively high annealing temperature (55°C) and a moderate number of amplification cycles (32). On the basis of Southern blotting of genomic DNA, we expected the strongest amplification for serovars sejroe, polonica, istrica, balcanica, dikkeni, and hardjo (type hardjobovis), whereas other serovars of serogroup sejroe were expected to give lower or no amplification. Under the conditions described the primers provided a specificity which fulfilled these expectations. A further increase in the annealing temperature did not significantly increase specificity. Even a careful selection of primers does not exclude the amplification of DNA that has little homology with the target DNA in hardjobovis. The intensity of the bands on agarose gels reflects only the degree of homology between primers and target DNA. Southern blots provide information on homology between amplified DNA and the clone from which the primers are derived. It is clear that the degree of specificity relies primarily on the primers, but so far, no primers have been selected that were entirely hardjobovis specific.

Our results show that PCR is potentially a valuable addition to the diagnostic methods in leptospirosis. Storage and presentation of urine samples do not appear to be critical (1). Preparation of the sample and performance of the assay can be done within one working day. The specificity of the assay is high and can even be increased by the application of new more-serovar-specific oligonucleotide primers. These qualities indicate that PCR may be useful in the diagnosis and epidemiology of leptospirosis.

## ACKNOWLEDGMENTS

We thank Pamela Wright for corrections of the English and Gerard Schoone for skillful assistance.

This study was supported by EEC grant TS2-0113-NL (GDF).

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