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Detection of Leptospiral DNA by PCR

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An *Eco*RI fragment (1.2 kb) which is highly conserved among *Leptospira interrogans* isolated in Korea was cloned into pBluescript vector from *L. interrogans* serovar lai WH20. The *Eco*RI fragment was sequenced, and a pair of primers (LP1 and LP2) was designed for PCR assay. PCR amplification of target DNA obtained from cultured *L. interrogans* showed that 274 bp could be detected when as little as 100 fg of leptospiral genomic DNA was used in the reaction mixture. No amplification of DNA was detected from DNA of *Leptospira biflexa* serovars patoc and sau paulo, *Borrelia burgdorferi*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium*. Amplification of 274-bp target DNA could be detected in DNA samples purified from 500 μl of blood collected from experimentally infected gerbils 2 days after infection, while antibodies to *L. interrogans* could be detected by the microscopic agglutination test 7 days after infection. The specificity and high sensitivity of the test provided valuable tools for the early diagnosis of leptospirosis.

Human leptospirosis caused by infection with strains of the Icterohaemorrhagiae serogroup of *Leptospira interrogans* has emerged as a potential problem in Korea (1–3). Some patients show signs of disseminated intravascular coagulation, which is followed by dyspnea and acute respiratory failure (6). Serological evidence of the widespread occurrence of leptospirosis has been reported (1–3), and *L. interrogans* serovars lai, hongchon, yeonchon, and canicola have been isolated from patients (13, 15).

Current diagnostic methods for leptospirosis usually depend upon demonstration of serum antibodies (4). The most commonly used serological test is the microscopic agglutination test (MAT). The MAT is highly sensitive, but it has some disadvantages. One of its main disadvantages is that the MAT is indirect. It does not recognize the presence of *L. interrogans* but rather recognizes the infected host's response. As such, the test does not indicate active infection. In Korea, nationwide vaccination has been performed since 1989. Therefore, interpretation of MAT results in Korea, especially in the early stage of the disease, is confusing. A further shortcoming of the MAT is that it is serovar specific. Therefore, it requires a large number of antigens in order to detect agglutinins to all of the different leptospiral serovars (4).

Recent advances in recombinant DNA technology have provided a new approach to the development of rapid and sensitive diagnostic tools. The PCR has come into increasing use for the diagnosis of infectious diseases caused by slowly growing or fastidious microorganisms (5). This report describes the identification of leptospiral oligonucleotide sequences which are highly conserved among *L. interrogans* strains isolated in Korea and their ability to amplify leptospiral DNA from cultured strains of *L. interrogans* or directly from blood samples obtained from experimentally infected gerbils.

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MATERIALS AND METHODS

Bacterial strains, cultural conditions, and plasmids. Seventeen serogroup reference strains of *L. interrogans* and 15 serovar reference strains of *L. interrogans* serogroup Icterohaemorrhagiae were obtained from the Centers for Disease Control, Atlanta, Ga. *L. interrogans* serovar lai WH20, HY2, HM4, 22R, and AP3; serovar canicola HS7; serovar hongchon 18R; and serovar yeonchon HM3; and strain 30R (serovar unknown), which were isolated in Korea, were supplied by the National Institute of Health, Seoul, Korea. *L. interrogans* serovar lai KH1, and KH2 were obtained from Konkuk University, Choongjoo, Korea. *L. interrogans* serovar lai HH14 was isolated and maintained by us for 3 years. *Escherichia coli* XL-1Blue (Stratagene, La Jolla, Calif.) was used for the preparation of all plasmids. pBluescript SK(−) (Stratagene) was used for cloning. Ellinghausen McCullough Johnson Harris (EMJH) medium was used for the culture of *L. interrogans* (5). Leptospires were harvested by centrifugation when each culture reached the log phase of growth (5×10^8 organisms per ml) as determined by nephelometer readings. Luria-Bertani medium was used for routine maintenance and transformation experiments. For all strains harboring recombinant plasmids, ampicillin (50 μg/ml) was added to the culture medium.

Genomic library construction and screening. To clone the DNA sequences which are conserved among all of the strains of *L. interrogans* isolated in Korea, two kinds of genomic libraries were made. One genomic library was constructed with DNA of *L. interrogans* serovar lai WH20, and the other one was constructed with DNA of *L. interrogans* serovar canicola HS7. Genomic DNAs of leptospires, extracted as described by Le Febvre et al. (8), were digested with *Eco*RI, ligated with *Eco*RI-digested pBluescript SK(−), and transformed into *E. coli* XL1Blue by the $CaCl_2$ method described by Maniatis et al. (9). About 2×10^3 to 3×10^3 transformants from each library were cultured on nitrocellulose papers (Schleicher and Schuell, Dassel, Germany) and three replicas were made from each plate. All replicas were incubated at 80°C in a vacuum oven and used for hybridization. One replica was screened with radiolabelled total genomic DNA of *L. interrogans* serovar lai WH20, another was screened with radiolabelled total genomic DNA of *L. interrogans* serovar canicola HS7, and another was

screened with radiolabelled total genomic DNA of *L. interrogans* serovar hongchon 18R as described by Maniatis et al. (9). Each replica was incubated in 50% formamide for 6 h at 42°C and hybridized with radiolabelled total genomic DNAs for 12 h at 42°C. The replicas were washed three times (5 min each) at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS), followed by two 30-min washes at 68°C in 0.1× SSC containing 0.1% SDS. Autoradiography was performed with Agfa-Curix XP films (AGFA, Mortsel, Belgium) for 12 h at −70°C. After the autoradiography, the transformants which hybridized with all three kinds of total genomic DNAs were chosen.

Southern blot hybridization. DNAs of leptospires were digested with *Eco*RI and electrophoresed in 1.0% agarose gels. The DNAs were transferred onto nitrocellulose papers with a vacuum transfer apparatus (TransVac; Hoefer Scientific Instruments, San Francisco, Calif.). Following transfer, the nitrocellulose papers were incubated at 80°C in a vacuum oven for an hour and hybridized with the radiolabelled probes under high-stringency conditions as described by Maniatis et al. (9).

DNA sequencing and primer design. The nucleotide sequences of both DNA strands of recombinant plasmid inserts were determined by the dideoxynucleotide chain termination method with a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio). The insert DNA of pLP4 was subcloned into pUC118 or pUC119, and both strands were sequenced with the dideoxynucleotide chain termination method. 5′-α-^{35}S-labelled dCTP (New England Nuclear, Boston, Mass.) and commercial M13 primers were used. One set of oligonucleotide primers (LP1 and LP2) was chosen for PCR. By using primers LP1 (5′-ATACAACTTAGGAAGAGCAT-3′) and LP2 (5′-GCT TCTTTGATATAGATCAA-3′) in the PCR test, an amplified 274-bp target could be obtained from all serovars of *L. interrogans* isolated in Korea.

DNA amplification by PCR. Ten nanograms of leptospiral DNA was placed in 99 μl of reaction mixture buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.01% Tween 20, 0.01% Nonidet P-40, 100 μM [each] deoxynucleoside triphosphate, 1 μM [each] primer) and was denatured at 95°C for 5 min. After the mixture was cooled to 72°C, 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was added and PCR tubes were placed in an Intelligent Heat Block (Hybaid). For specificity analysis, 100 ng (each) of *Staphylococcus aureus*, *E. coli*, *Salmonella typhimurium*, *Leptospira biflexa* serovars patoc and sau paulo, and *Borrelia burgdorferi* DNA was used. The PCR profile was as follows: 1 min of template denaturation at 95°C, 30 s of primer annealing at 55°C, and 45 s of primer extension at 72°C, for a total of 35 cycles, with a final extension at 72°C for 5 min. For the sensitivity analysis, a 10-fold dilution of purified leptospiral DNA was made, and the DNAs were subjected to PCR amplification as described above. PCR amplification products were detected and identified by visualization of the bands of the expected size on agarose gels.

Experimental animals. Peripheral blood samples from gerbils infected with 0.2 ml of *L. interrogans* serovar lai KH1 (optical density at 550 nm of 0.04) were collected daily. The 0.5 ml of the collected blood was mixed with the same volume of Alserver solution (10 mM dextrose, 27 mM sodium citrate, 2.6 mM citric acid, 70 mM sodium chloride) and centrifuged at 3,000 × *g* for 2 min. The supernatants were harvested and subjected to centrifugation at 15,000 × *g* for 15 min to obtain leptospiral pellets. The pellets were suspended with 400 μl of Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0], 50 mM EDTA) containing SDS (0.5%) and proteinase K (50 μg/ml) and were incubated at 45°C for 3 h. After incubation, the lysates were deproteinized with phenol-chloroform and DNAs were precipitated with ethanol. The precipitated DNAs were suspended in sterile distilled water and stored at −70°C until use. PCR results were compared with those obtained by the MAT.

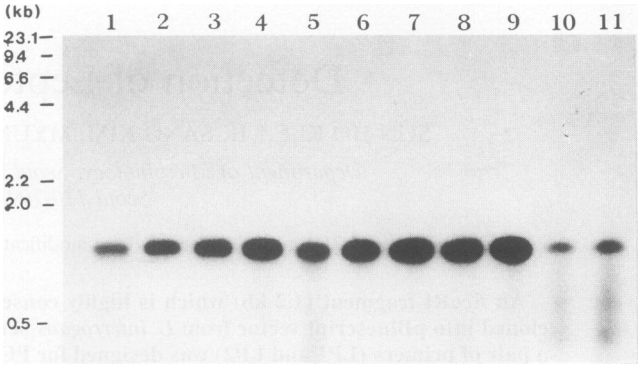

FIG. 1. Southern blot hybridization of *Eco*RI-digested genomic DNAs of nine strains of *L. interrogans* isolated in Korea and serovar reference strains. Lanes: 1, serovar lai; 2, WH20; 3, HY2; 4, HM4; 5, HH14; 6, 22R; 7, HM3; 8, 18R; 9, 30R; 10, HS7; 11, serovar canicola.

MAT. The titer of antibody in each serum sample collected from gerbils infected with *L. interrogans* serovar lai KH1 was determined by using *L. interrogans* serovar lai WH20 as an antigen as described by Cole et al. (4).

Nucleotide sequence accession number. The sequence data reported here have been submitted to the GenBank data bank under accession no. L13921.

RESULTS

Selection of primers. Twelve clones carrying pBluescript vector containing leptospiral DNA insert were isolated from the *L. interrogans* serovar lai WH20 genomic library (8 clones) and the *L. interrogans* serovar canicola HS7 library (4 clones) (data not shown), which hybridized with radiolabelled total genomic DNA of *L. interrogans* serovars lai WH20, canicola HS7, and hongchon 18R. By analysis of the sizes of the insert DNAs of these 12 clones, it was found that pLP4 from the WH20 genomic library and pLP12 from the canicola library have the same size of insert DNA (1.2 kb). It was also found that the insert DNA of pLP4 hybridized strongly with that of pLP12 by dot blot hybridization. Therefore, pLP4 was selected for the analysis of hybridization patterns with nine Korean isolates, the reference strain for serovar lai, and the reference strain for serovar canicola. The nine Korean isolates included all of the serovars reported in Korea up to the time of the study. pLP4 hybridized with a 1.2-kb *Eco*RI-digested DNA fragment of all Korean isolates tested and the reference strains for serovars lai and canicola (Fig. 1). These results suggested that the insert DNA of pLP4 could be used for the detection of *L. interrogans* which prevailed in Korea. The confirmed DNA sequence of part of pLP4 and the primer orientation are shown in Fig. 2. The GenBank DNA data bases did not reveal significant homology with any other known sequences. The LP1 and LP2 primer pair is situated outside the 274-bp fragment (Fig. 2). The ^{32}P-labelled 274-bp probe hybridized with the PCR product of pLP4 and the insert DNA of pLP4 (Fig. 3). To confirm that this primer set could amplify the target sequence from DNAs of *L. interrogans* isolated in Korea, DNA amplification was performed with 12 Korean isolates; the 274-bp amplification product could be observed in all of the

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          10         20         30         40         50         60
5'- ATACAACTTA GGAAGAGCAT ATCATCTGGA AAAAAAATAT CAGAAATTCC TTATCCAATT
    LP1   70         80         90        100        110        120
    TGAATATGCA AGCTCTGAAA TTCCTGAATA TTATAGAACC TTTATTCATT TAGGAACCGT
         130        140        150        160        170        180
    TATACGAAAT CATGAGAGAA CCGATCAATG CGACGATTTT ATGGAAAAAT GCGGTTAGTC
         190        200        210        220        230        240
    TTAATAAATT TCATACGGAA GCGTTACTAC TTTTAGCAGA CCACTACATT CGAACCGATC
         250        260        270
    TCAGAAATCG TGCATTGATC TATATCAAAG AAGC -3'
                   LP2
```

FIG. 2. Nucleotide sequence of the target DNA and the position of the LP1 and LP2 primer set (underlined).

strains tested (Fig. 4). To further determine the detection range of this PCR method, 17 serogroup reference strains, 15 serovar reference strains in serogroup Icterohaemorrhagiae, and 6 serovar reference strains in serogroup Canicola were tested. The 274-bp amplification product could be found in 14 serogroup reference strains (Fig. 5A), 11 serovar reference strains in serogroup Icterohaemorrhagiae, and 3 serovar reference strains in serogroup Canicola (Fig. 5B).

PCR sensitivity and specificity. To determine the sensitivity of the PCR assay, purified leptospiral DNAs were serially diluted from 100 pg to 1 fg and amplified. The smallest amount of leptospiral DNA that could be amplified by this PCR method was 100 fg (Fig. 6A). We also tested the ability of this pair of primers to amplify the DNAs of other spirochetes (*B. burgdorferi* and the *L. biflexa* serovars patoc and sau paulo). No amplification products from the DNAs of these spirochetes were detected (Fig. 6B). No amplification from the DNA of *S. aureus*, *E. coli*, and *S. typhimurium* was detected (data not shown).

Animal studies. Blood samples from gerbils that were experimentally infected with 10^5 cells of *L. interrogans* serovar lai KH1 were collected, and PCR amplification and the MAT were performed. The blood samples were collected with 1-day intervals from just after infection to 8 days postinfection, when all infected gerbils died. The infected gerbils developed clinical signs of infection (i.e., fever >40°C, coarseness of fur, and reduced activity) from 5 days postinfection. The PCR products could be detected 2 days postinfection, while antibodies to *L. interrogans* could be detected by MAT (Table 1) 7 days postinfection.

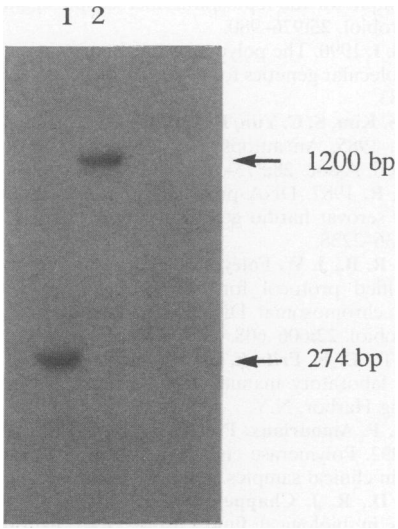

FIG. 3. Southern blot hybridization of *Eco*RI-digested pLP4 and PCR product with ^{32}P-labelled PCR product. Lane 1, PCR product from pLP4 clone; lane 2, *Eco*RI-digested pLP4.

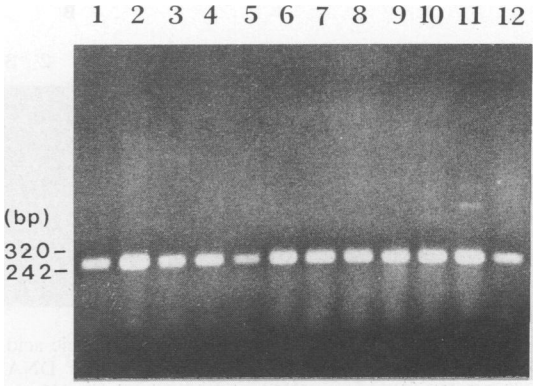

FIG. 4. PCR amplification of the target DNA from 12 strains of *L. interrogans* isolated in Korea. Lanes: 1, WH20; 2, HY20; 3, HM4; 4, KH1; 5, KH2; 6, HH14; 7, 22R; 8, AP3; 9, HM3; 10, 18R; 11, 30R; 12, HS7.

DISCUSSION

Current diagnostic methods that do not require culture, such as the MAT (4) and nonamplified DNA probe hybridization, are available (7, 11, 12, 19) for the diagnosis of leptospirosis.

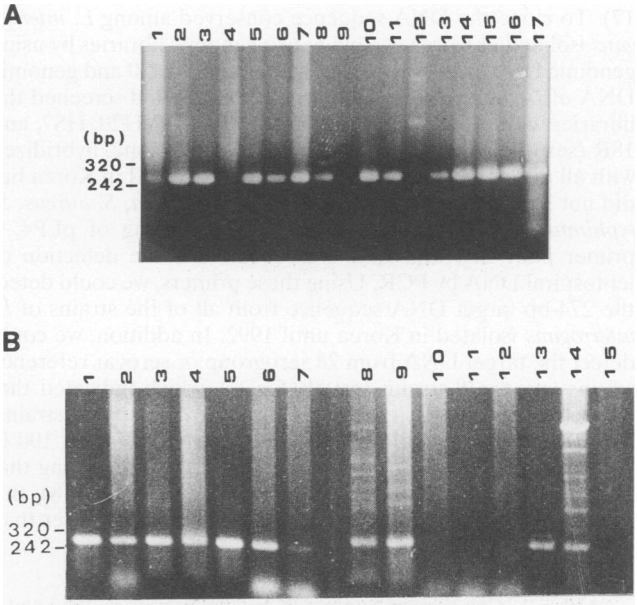

FIG. 5. PCR amplification of 274-bp target DNA from reference strains of *L. interrogans*. (A) DNAs from 17 serogroup reference strains. (B) DNAs from 15 serovar reference strains in serogroup Icterohaemorrhagiae. (A) Lanes: 1, australis; 2, autumnalis; 3, ballum; 4, balcanica; 5, canicola; 6, celledoni; 7, cynopteri; 8, djasiman; 9, grippotyphosa; 10, hebdomadis; 11, icterohaemorrhagiae; 12, javanica; 13, panama; 14, pomona; 15, pyrogenes; 16, hardjo; 17, tarassovi. (B) Lanes: 1, birkini; 2, copenhageni; 3, gem; 4, icterohaemorrhagiae; 5, lai; 6, mankarso; 7, monymusk; 8, mwogolo; 9, naam; 10, ndahambukuje; 11, ndambari; 12, sarmin; 13, smithi; 14, tonkini; 15, weaveri.

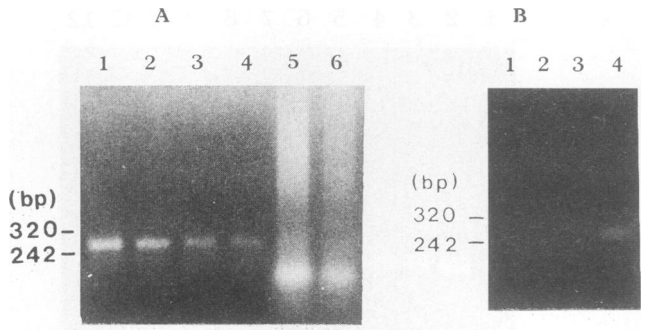

FIG. 6. Agarose (1.0%) gel electrophoresis of nucleic acid amplification products from purified and serially diluted DNA of *L. interrogans* KH1 (A) and from DNA of *B. burgdorferi*, *L. biflexa* serovar patoc, and *L. biflexa* serovar sau paulo (B). (A) Lanes 1, 2, 3, 4, 5, and 6 represent products from 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg of *L. interrogans* KH1, respectively. (B) Lanes 1, 2, 3, and 4 represent products from *B. burgdorferi*, *L. biflexa* serovar patoc, *L. biflexa* serovar sau paulo, and *L. interrogans* KH1, respectively.

The MAT is sensitive, but because of the antigenic heterogeneity of *L. interrogans*, it requires a large number of serovars as antigens. Also, it could not be applied in the early stage of the disease when the antibody to *L. interrogans* is not present or, if present, is at a low level in peripheral blood. The DNA hybridization test is also effective, but this method does not approach the sensitivity possible with the PCR (10). In Korea, some patients with leptospirosis showed acute respiratory distress symptoms and died in the early stage of the disease. Therefore, early diagnosis of the disease is very important for the treatment of leptospirosis in Korea.

L. interrogans is known to be genetically heterogeneous (14, 17). To clone the DNA sequence conserved among *L. interrogans* isolated in Korea, we made two genomic libraries by using genomic DNA of *L. interrogans* serovar lai WH20 and genomic DNA of *L. interrogans* serovar canicola HS7 and screened the libraries with radiolabelled genomic DNA of WH20, HS7, and 18R (serovar hongchon). pLP4, the resulting clone, hybridized with all of the DNAs from *L. interrogans* isolated in Korea but did not hybridize with the DNAs from *L. biflexa*, *S. aureus*, *S. typhimurium*, and *E. coli*. By DNA sequencing of pLP4, a primer pair, LP1 and LP2, was chosen for the detection of leptospiral DNA by PCR. Using these primers, we could detect the 274-bp target DNA sequence from all of the strains of *L. interrogans* isolated in Korea until 1992. In addition, we could detect the target DNA from 28 serogroup or serovar reference strains among 38 strains tested (Fig. 4), which indicated that this sequence is highly conserved among *L. interrogans* strains. This PCR method could amplify the target DNA from 100 fg of DNA of *L. interrogans* serovar lai WH20. Considering that the size of the leptospiral genome is about 5,000 kb (18), this result indicated that this PCR method could detect fewer than 10 copies of leptospiral genome. The sensitivity of this PCR is 10 to 50 times higher than that of the DNA hybridization method (16). We could not amplify the target DNA from 4 serovar reference strains in serogroup Icterohaemorrhagiae among 17 serovar reference strains tested and 3 serovar reference strains in serogroup Canicola among 6 reference strains tested. We cannot explain this result. However, if we consider the results reported by Yasuda et al. that serogrouping of *L. interrogans* did not correlate well with genetic relatedness and that there was little genetic homology between the serovars sarmin and icterohaemorrhagiae (17), although they are classified into same serogroup, Icterohaemorrhagiae, it could be presumed that the serovars ndahambukuje, ndambari, sarmin, and weaveri are genetically different from other serovars in the serogroup Icterohaemorrhagiae.

TABLE 1. Comparison between PCR amplification method and antibody detection by MAT with experimentally infected gerbils

Test	No. positive/no. of gerbils tested at postinfection day[a]:								
	0	1	2	3	4	5	6	7	8
MAT	0/3	0/3	0/3	0/3	0/3	0/3	0/3	3/3	3/3
PCR amplification	0/3	0/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3

[a] Day postinfection when blood samples were collected.

The applicability of the PCR method for the clinical diagnosis of leptospirosis was evaluated with blood samples from gerbils experimentally infected with *L. interrogans*. This PCR method could detect leptospiral DNA from blood collected 2 days postinfection, while antibody to *L. interrogans* could be detected from the blood collected 7 days postinfection. This result showed that the PCR method had advantages over the MAT in the early diagnosis of leptospirosis.

In conclusion, on the basis of its sensitivity and specificity, this PCR method should be useful as a tool for early diagnosis of leptospirosis in Korea irrespective of serovar.

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